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13. ABSTRACT ***(Maximum 200 Words)***

Behavioral, neurochemical, and immunocytochemical studies are characterizing the possible role of insecticide exposure in the etiology of Parkinson's disease as it may relate to Gulf War Syndrome. Chlorpyrifos (CP) and/or permethrin (PM) were given 3 times over a two week period by injection (CP subcutaneous and PM intraperitoneal), with or without a single dose of the Parkinsonian neurotoxin, MPTP (20-30 mg/kg, intraperitoneal). PM upregulates dopamine transporter (DAT) expression with a 28 day time course and at doses as low as 0.2 mg/kg, while CP has little effect. Data from western blots of DAT protein correlate well with measurements of [^{3}H]GBR12935 binding, a ligand for the DAT. When applied as CP + PM + MPTP, expression of the DAT is significantly decreased, which is consistent with neuronal injury. In addition, this is probably a synergistic interaction, since MPTP and CP are inactive alone, and PM upregulates the DAT. Similar experiments using labeled quinuclidinyl benzilate as a ligand for muscarinic cholinoceptors observed no significant interaction, suggesting that there is a lack of a significant synergistic effect of these compounds on striatal cholinergic pathways. Because technical permethrin is a mixture of four stereoisomers, further work is underway to characterize the isomer(s) of permethrin involved in DAT upregulation.

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INTRODUCTION

This study is focused on the neurotoxic actions of the insecticides permethrin (PM) and chlorpyrifos (CPF) as they relate to the development of Parkinson's Disease (PD). These compounds possess properties that could damage the nigro-striatal system, which is the primary brain lesion in PD (Bowman and Rand, 1980). The research is assessing the ability of each compound alone, or in combination, to directly induce neurochemical or neuropathological hallmarks of PD. In addition, since PD is hypothesized to have a multifactorial etiology (Siderowf, 2001), these compounds are also being tested for their ability to synergize the actions of the established Parkinsonian neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). This approach will determine any ability of the insecticides to accelerate or intensify idiopathic disease processes. Experiments are performed on the C57BL6 black mouse, which when given MPTP is a valid rodent model for the development of PD (Heikkila and Sonsalla, 1992). For each treatment group, effects consistent with metabolic insult and changes in cholinergic and dopaminergic neurotoxicity in the striatum are measured. Cell stress in striatal nerve terminals is evaluated by measurements of mitochondrial function. Other neurochemical studies measure effects specific to the dopaminergic pathways in the striatum, including the amounts of dopamine and its metabolite 3,4-dihydroxyphenylacetic acid, as well as the ability of isolated nerve terminals to transport dopamine. Because of the anticholinergic effect of chlorpyrifos, we also measure acetylcholinesterase activity, and the density/function of muscarinic and nicotinic receptors following insecticide treatment. Neuropathology studies are focused on any gross changes in immunocytochemical markers for glial fibrillary acid protein (GFAP). Other antibody labeling studies will assess effects specific to the dopaminergic system, including antibody labeling of tyrosine hydroxylase and the dopamine transporter (DAT). These studies represent a unique combination of research approaches and will provide a comprehensive and integrated evaluation of the possible Parkinsonian or neurodegenerative effects of these insecticides.

BODY

The experiments for the fourth year (a no-cost extension) were, in part, completion of studies related to Objective #1, which is to characterize any effects on biomarkers of PD over a range of doses of PM and CPF. Other studies have addressed objectives related to possible synergistic interactions of toxicants (Objective #2), as well as the reversibility of effects on various biomarkers by assessing effects at various times after the last treatment (Objective #3). Supplemental funding was provided by the sponsor to look at isomers of permethrin responsible for DAT upregulation (funding period: 2/1/03-1/31/04). Thus, this report is not the final one for the whole project. A significant amount of time this year was allocated to further data analysis and paper writing (see Appendix). Experimental results for year four of the project are organized by alphabetical listing of biomarker assessments, as given in the amended grant proposal and in the annual reports for previous years.

Treatment of mice in year four for studies of insecticides alone, and insecticides with MPTP, was performed as described in the proposal, and is illustrated in Figures 1 and 2, respectively. Treatment groups for the procedure given in Figure 1 were controls, PM, CPF, or PM+CPF. Treatments for the procedure given in Figure 2 were controls, MPTP, MPTP+PM, MPTP+CPF, or MPTP+PM+CPF. In addition, our previous biomarker analyses were nearly all performed 24 hr after the last treatment, and we have expanded these results to include time points 2 and 4 weeks after treatment to evaluate the persistence of effect (Figs. 1 and 2).

One of the observations we reported previously was greater than expected mortality in treatments with MPTP at 30 mg/kg, which suggested that lower doses needed to be used. No lethality occurred at the doses of MPTP used in the past year (20 or 30 mg/kg).

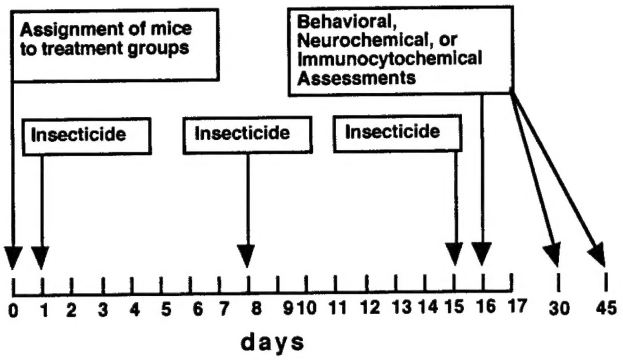


Figure 1. Treatment regime for studies with insecticides alone (PM, CPF) or in combination (PM+CPF).

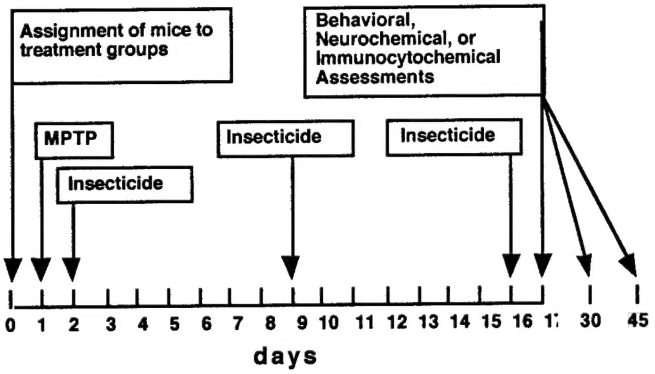


Figure 2. Treatment regime for studies of insecticides in combination with MPTP (MPTP, MPTP +PM, MPTP + CPF, MPTP+PM+CPF). Animals receiving MPTP alone were also given insecticide vehicle.

a. Assess toxicant effects on dopamine titers and turnover by measuring the dopamine and 3,4-dihydroxyphenyl acetic acid (DOPAC) content of the striata from treated mice.

No experiments of this type were run in the last year.

b. Assess effects on the density and kinetic properties of dopamine transporters in striatal synaptosomes from treated mice.

One of our most significant findings is the demonstration that DAT protein is upregulated by PM in measures of dopamine transport, [^{3}H]GBR 12935 binding, and DAT protein in Western blots. No studies of dopamine uptake were performed during the past year. However, because it is appropriate for comparison with new results, some older data is presented below (Fig. 3). In these experiments, we found that a dose of 1.5 mg/kg PM significantly increased dopamine uptake by striatal synaptosomes, but at higher doses (*e.g.*, 200 mg/kg) uptake was decreased to levels less than that of controls (Fig. 3).

Methods: For both dopamine uptake and radioligand binding studies of the DAT, we prepared striatal synaptosomes *ex vivo* from treated mice using the methods of Bloomquist *et al.* (1994). For labeling of dopamine uptake sites, we used an equilibrium binding assay with the established DAT radioligand [^{3}H]GBR12935 (Horn, 1990). Although others have found fault with this ligand in some assay systems (Pristupa et al., 1999), it saturates well in native tissue preparations (Horn, 1990 and last year's report). Briefly, striatal tissues were homogenized in ice cold Krebs-Ringer's-HEPES (KRH) buffer (118 mM NaCl, 4.8 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM $MgSO_4$, 20 mM HEPES, pH=7.4). Striata were then centrifuged at 18,000 x g and resuspended for one hour in distilled water. The tissues were again centrifuged at 18,000 x g, resuspended in cold KRH buffer, and stored at -70 °C until use. For the assay, samples were thawed and run in duplicate in the absence and presence of 0.5 μM GBR 12909. To measure total binding, [^{3}H]GBR 12935 (100, 50, 25, 12.5, and 6.25 nM) was incubated with KRH buffer and tissue for 2 hours at 4 °C. The same

procedure was used to measure non-specific binding with the exception that GBR 12909 was added to the reaction mixture. The reactions were stopped by the addition of cold KRH buffer, and the contents of each reaction tube was filtered through 25 mm Whatman GF/B filters pre-soaked in 0.1% BSA. Filters were then washed 3 times with cold KRH buffer to remove unbound radioactivity. The filters were soaked overnight in Scintiverse E, and total counts were measured on a Beckman LS 6500 liquid scintillation counter. Aliquots of [^{3}H]GBR 12935 concentrations (100, 50, 25, 12.5, and 6.25 nM) were added to Scintiverse E cocktail to calculate the exact [^{3}H]GBR 12935 concentrations in each reaction mixture. Following protein determination (Bradford, 1976), nonlinear regression to isotherm plots was used to determine B_{max} and K_d using Prism™ (GraphPad Software, San Diego, CA). K_d and B_{max} values of different treatments were compared by T-test or ANOVA, as appropriate.

Western blot analysis of DAT and other proteins was approached by the following general procedures. Murine striatal synaptosomal proteins (2-10 μg protein per lane, depending on the protein to be measured) were separated on a 10 % SDS-PAGE gel and transferred to a nitrocellulose membrane (Bio-Rad). The membrane was subsequently incubated in 4% dry milk, followed by overnight incubation in a rat monoclonal primary antibody (Chemicon) at 4° C. Primary antibody was typically diluted to 1:1000. The blots were then incubated in a peroxidase-linked secondary antibody (anti-rat; Sigma Chemical) for one hour at room temp. Blots were developed using the chemiluminescence detection method (Amersham Pharmacia), and exposed to photographic film (ECL hyperfilm, Amersham-Pharmacia Corp.) for varying lengths of time (5-20 sec). Protein bands on the film were quantitated using the Kodak EDAS 290 system (Eastman Kodak Co.). Protein content was determined by the method of Bradford (1976).

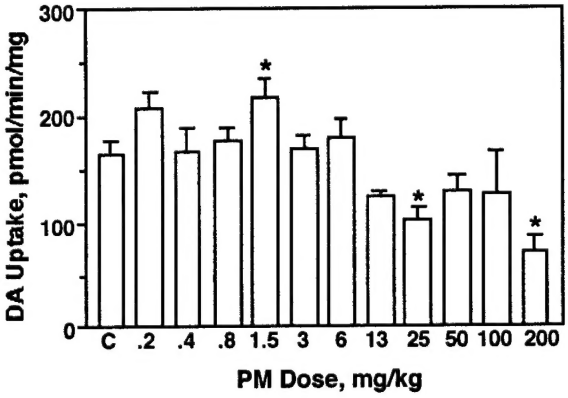


Fig. 3. Bar graph of the effects of PM on V_{max} for dopamine uptake. Bars represent means of three determinations with error bars equal to the SEM in this and all subsequent figures. Asterisks indicate effects significantly different from control (T-test, $p<0.05$). Data taken from Karen *et al.* (2001).

Results and Discussion: A significant amount of effort in the past year was expended in evaluating the levels of DAT protein in different treatment groups and whether it correlated with the binding of [^{3}H]GBR 12935. These studies were related to all three major technical objectives of the project. Additional computations and statistical analysis are shown in the following figures for 0.8 and 1.5 mg/kg doses of PM (Figs. 4-5), preliminary forms of which were shown in last year's report. Densitometry scans of Western blots (Fig. 4) showed an increase in DAT protein that correlated with the increase in dopamine uptake observed previously around 1.5 mg/kg PM. Of course, the optimal dose for observing increased uptake varies a bit between different cohorts of mice, and represents experimental variability. In the experiment shown in Figure 4, increased DAT protein was apparent in one day at 0.8 mg/kg PM, with the 1.5 mg/kg dose significantly elevated by 28 days. Changes in DAT protein in Western blots were correlated with the B_{max} value for GBR

binding, including its time course (Fig. 5). No significant changes in K_d values were noted in any of the experiments. The slow time course and persistence of the effect on DAT was unexpected, since permethrin is short lived *in vivo* (Anadon *et al.*, 1991), so it must be setting in motion a signaling pathway or other condition that is much more persistent.

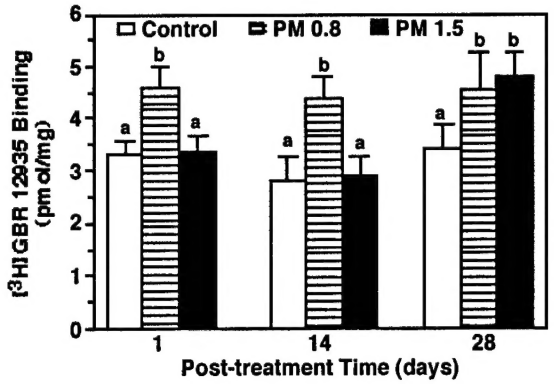


Fig. 4. Time course experiments of [^{3}H]GBR 12935 binding in C57Bl/6 mice treated with 0.8 mg/kg or 1.5 mg/kg permethrin. Letters denote results of ANOVA with Student-Newman-Keuls means separation test, where treatments within a particular time period (1, 14, or 28 days) are significantly different ($p < 0.05$) when labeled by different letters.

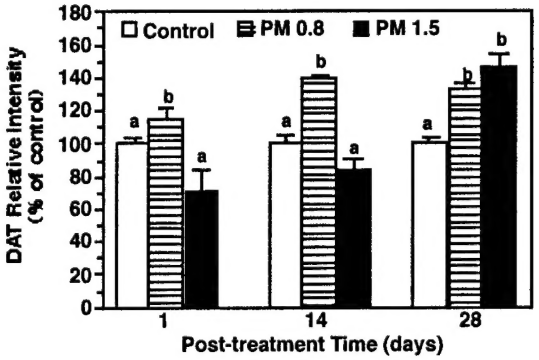


Fig. 5. Time course experiments of DAT western blot densitometry in C57Bl/6 mice treated with 0.8 mg/kg or 1.5 mg/kg permethrin. ANOVA was performed on the raw data before calculation as % of control. Statistical analysis as described for Fig. 4.

Previous studies of [^{3}H]GBR12935 binding and DAT western blots in PM-treated C57 mice (100 and 200 mg/kg) showed no difference from controls 24 hr after the last treatment, even though dopamine uptake was reduced at these doses (Fig 3). Taken together, these observations suggested that other mechanisms besides a change in density of the DAT was responsible for the reduced uptake, such as enhanced leakage from the synaptosomes due to cytotoxicity. Pyrethroids are known to stimulate secretion of transmitters (Kirby *et al.*, 1999), and there may also be a PM-induced effect on mitochondrial respiration (Gassner *et al.*, 1997). We have observed reduced complex I activity (MTT reduction assay) in *ex vivo* striatal synaptosomes from mice given high doses of PM (Karen *et al.*, 2001). In longer term studies (Fig. 6) there is recovery of ability to upregulate DAT 2-4 weeks after the last treatment of 200 mg/kg PM. These findings suggest that whatever cytotoxicity occurred was reversible.

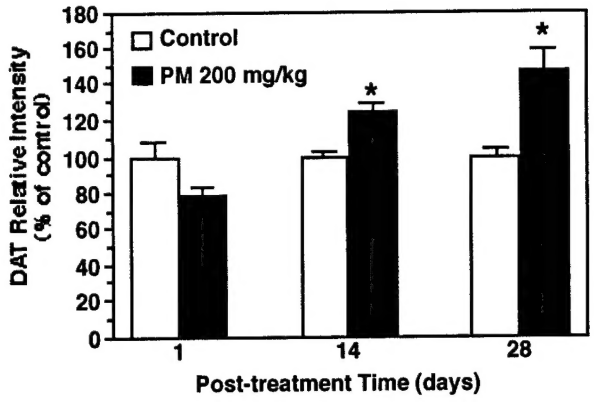


Fig. 6. Time course studies using densitometry of DAT western blots of striatal synaptosomes from mice treated with 200 mg/kg PM. Raw data were analyzed by T-test before calculation as % of control, and bars labeled by an asterisk for a particular time point (1, 14, or 28 days) are significantly different ($p < 0.05$).

Additional studies (Fig. 7) were performed to identify the no observable effect level (NOEL) for DAT upregulation using the standard treatment paradigm (Fig. 1). These experiments found that DAT protein was significantly upregulated at doses as low as 0.2 mg/kg PM. This dose was effective as early as 1 day post-treatment, and did not increase thereafter.

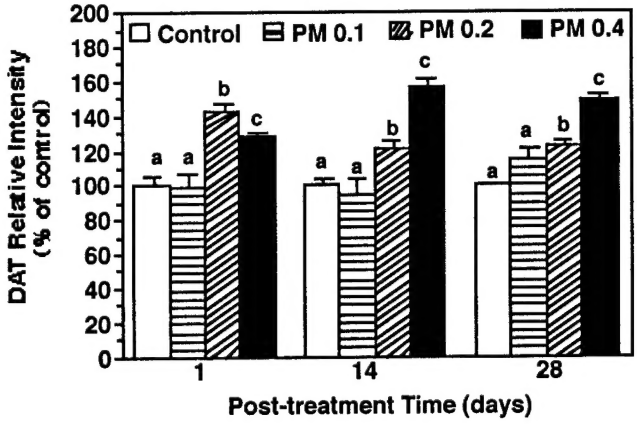


Fig. 7. Time course studies using densitometry of striatal synaptosomes on DAT western blots from mice treated with either 0.1, 0.2, or 0.4 mg/kg PM. Statistical analysis as described for Fig. 4.

The second treatment paradigm was related to Objective #2, and exposed mice to mixtures of toxicants to evaluate any synergistic or antagonistic effects. In previous studies, we found at best small synergistic effects of insecticides with MPTP in measures of striatal dopamine content (reports from 2000 and 2001) at the highest dose of MPTP we used (30 mg/kg). However, these measurements were performed at only one time point (1 day). Additional studies in the past year used DAT expression as a biomarker in combination treatments assessed at 14 and 28 days (Figs. 8-9). This approach was used because western blot analysis can be performed on smaller treatment groups of mice than dopamine analysis by HPLC, so we conserved experimental animals. At 14 days post-treatment (Fig. 8), there was no statistically significant change in DAT expression for any compound given alone. However, there was a significant increase in striatal DAT protein when MPTP was combined with PM, and in the triple treatment group.

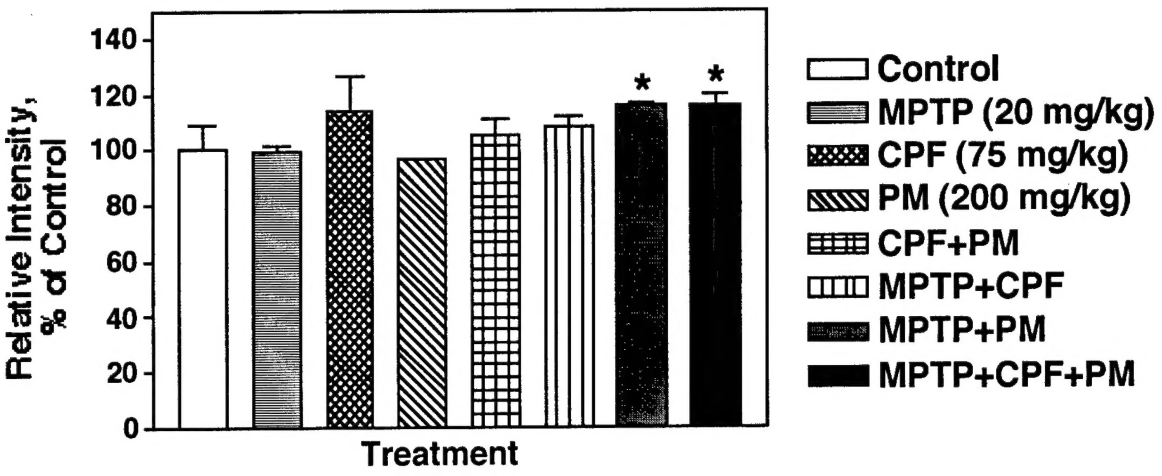


Fig. 8. DAT expression two weeks after dosing with insecticides alone or in combination with each other or MPTP. Letters denote results of a T-test applied to the data before expression as % of control. Bars labeled with an asterisk are significantly different from control ($p<0.05$).

More significant effects were observed 28 days after the last treatment (Fig. 9). At this time point, there was a significant increase in a the double treatment groups. This result is not surprising for treatments having 200 mg/kg PM, which alone increases DAT at 28 days (Fig. 6). The additional presence of CPF or MPTP in the double treatment groups (PM+CPF and PM+MPTP) caused no further increase in DAT expression. There was a synergistic increase in DAT protein in the CPF+MPTP group, where either compound alone was inactive. The most significant finding is the reduced DAT protein in the triple treatment group, which would be consistent with neurotoxicity. If so, we would expect to see a reduction in dopamine titers and impaired behavior in this group.

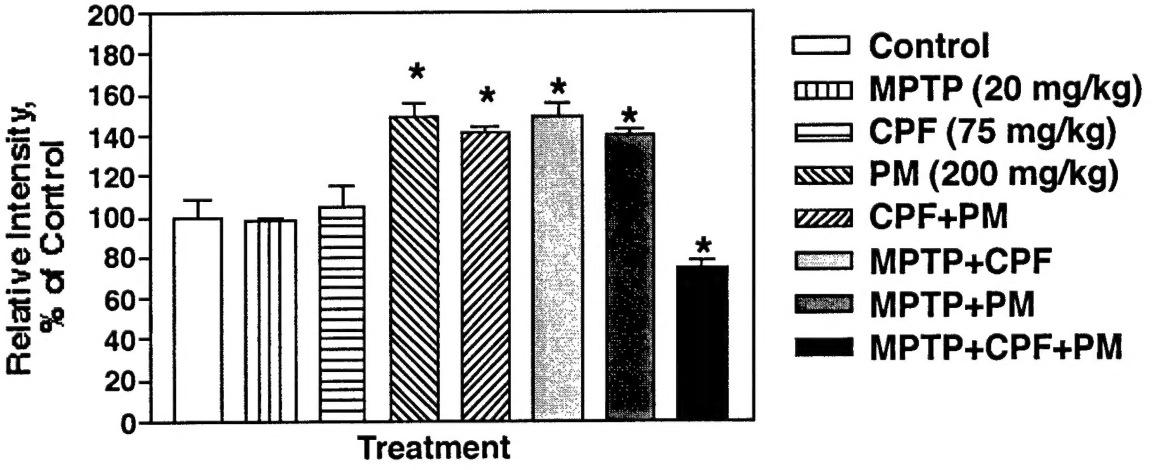


Fig. 9. DAT expression four weeks after dosing with insecticides alone or in combination with each other or MPTP. Letters denote results of a T-test applied to the data before expression as % of control. Bars labeled with an asterisk are significantly different from control ($p<0.05$).

c. Compare the extent of toxin-dependent actions on mitochondrial function in striatal synaptosomes by measuring thiazolyl blue dehydrogenase activity.

No experiments of this type were run in the past year.

d. Search for anatomical evidence of general neurotoxicity within light microscopic preparations of the nigro-striatal system by examining glial fibrillary acidic protein (GFAP) immunoreactivity as a marker for gliosis.

e. Search for anatomical evidence of general neurotoxicity within specific dopaminergic components of the nigro-striatal system using immunocytochemical staining for the catecholamine-synthesizing enzyme tyrosine hydroxylase (TH).

f. Confirm whether functional changes in dopamine transport are due to fluctuating levels of dopamine transporter (DAT) protein using immunocytochemical identification.

Methods: These biomarkers are dealt with together since fixation, sectioning, staining and analysis of the tissue is similar for all. Detailed explanation of the methods involved can be found in the Appendix, paper by Pitman *et al.* In the previous reporting period (1/2001-1/2002) we presented data regarding the possibility of synergism between CPF (50 mg/kg) and PM (200 mg/kg), when administered together, in producing neurotoxic effects upon striatal dopaminergic terminals. We also introduced the use of GFAP immunostaining as a more subtle indicator of nigrostriatal nerve terminal insult.

In the present reporting period (1/2002-1/2003) we spent a significant amount of time attempting to improve the quality of our immunohistochemical staining for TH and GFAP. This involved testing antibodies from a number of suppliers at a number of different concentrations. In addition, a new graduate student (Ms. Celia Dodd) assumed responsibility for the immunohistochemical studies. This necessitated instruction for and practice with the immunohistochemical technique. She is now proficient in the necessary techniques and has initiated immunohistochemical studies of potential synergistic effects of the insecticides PM (200 mg/kg) and CPF (50 mg/kg) upon the dopaminergic terminal destruction induced by MPTP (30 mg/kg). A brain section from each dosing group, taken from a matched set of 4 mice, can be placed on a single microscope slide and run through the immunohistochemical procedure under identical tissue processing conditions. This is done for 3 separate, fixed, striatal loci within each brain: two loci for antibody treatment and one for omission control. A second set of immediately adjacent sections is prepared for staining with a second antibody (either TH or GFAP). Although 4 sections on a given slide is the upper limit of our technical capability, this method allows direct comparison of brain sections from a matched quartet of mice, from each dosing group, that have been processed identically.

Results and Discussion: The dosing and tissue processing for the experiment examining synergism between PM and MPTP, upon TH and GFAP immunoreactive striatal neuropil, has been completed, but the analysis is ongoing and could not be finished in time for this report. We are about to begin the dosing for additional experiments.

In the past year we prepared and submitted a paper to the International Journal of Toxicology regarding the effects of low and high doses of PM alone on striatal immunohistochemical staining for DAT, TH and GFAP. The data contained in the paper have been presented in previous progress reports and a copy of the paper, currently under review, is attached (Pitman *et al.*, appendix).

g. Explore toxicant effects on open field/rearing frequencies and pole climbing behaviors and search for correlations between behavioral impairment and neurochemical effects.

Because we focused on neurochemical endpoints during the past year to use fewer mice, we have no new behavioral data to report. An important future issue is whether the PM + CPF + MPTP group will show significant behavioral impairment 28 days after treatment, consistent with the reduction in DAT we observed (Fig. 9).

h. Determine the extent of acetylcholinesterase inhibition following treatment with toxicants for comparison with other behavioral and neurochemical effects.

Methods: We used the classical method of Ellman *et al.* (1961) to determine acetylcholinesterase activity in brain tissue from treated mice. The assay measures the reaction of 5,5'-dithiobis-2-nitrobenzoic acid and thiocholine, when acetylthiocholine is used as the enzyme substrate. Although V_{max} and K_m values for acetylcholinesterase activity were originally planned, standard measurement of cholinesterase involved a single substrate concentration at an incubation time in the linear range of activity, for simplicity. We used a substrate concentration of 400 μM and an incubation time of 3 min, as established in pilot studies. Tissue dilutions were prepared by combining tissue homogenates with 3 parts 0.1 M $NaPO_4$ buffer (pH=7.8). Absorbance values were converted to moles using the extinction coefficient reported by Ellman *et al.* (1961). Data were analyzed by ANOVA and T-test as appropriate.

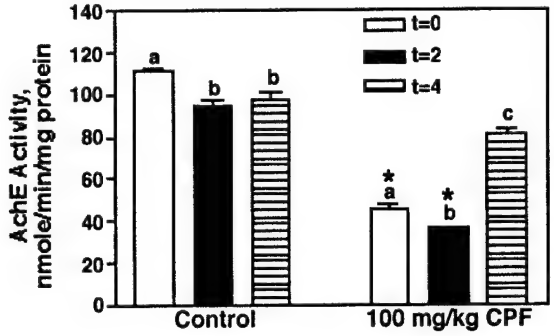


Fig. 10. Acetylcholinesterase activity in the cortex of mice treated with CPF. Letters indicate results of ANOVA and Student-Newmann-Keuls means separation for controls or CPF-treated tissue for the three time points analyzed, where t=0 (1 day), t=2 (2 weeks), and t=4 (4 weeks) indicate time of assay after the last treatment. Bars labeled by different letters are significantly different ($p<0.05$). Asterisks indicate significant difference ($p<0.05$) between control and CPF-treated mice at a given time point (paired T-test).

Results and Discussion: In previous studies, we observed approximately 15, 50, and 80% inhibition of cholinesterase activity at doses of CPF of 25, 50, and 100 mg/kg, respectively, and the extent of inhibition was nearly identical (within a few percent) in either striatal or cortical tissue. We determined the cortical acetylcholinesterase activity at additional time points (1, 14, or 28 days), for correlation with measures of QNB binding (see below). In these studies, inhibition at 100 mg/kg CPF was initially about 60%, and was stable for 2 weeks following the last dose (Fig. 10). By t=4 weeks, the activity had substantially recovered, and was 18% inhibited, although this level of activity was not quite significantly different from the control ($p=0.0583$). We do not ascribe any particular biological significance to the slightly reduced activity in the controls at t=2 and t=4, although the ANOVA indicated statistical significance. The Ellman assay has a high level of precision and is quite reproducible, resulting in small degrees of variability typically showing up as significant differences between or within experimental groups.

i. Define any toxicant-induced changes in cholinergic receptor density or function with respect to agonist-induced dopamine release from striatal synaptosomes.

Methods: This last objective of the research actually contains several separate neurochemical measurements. We have established methods for radioligand binding studies involving [^{3}H]quinuclidinylbenzilate ([^{3}H]QNB) and [^{3}H]epibatidine, with epibatidine binding adapted from the procedures of Houghtling *et al.* (1995). In addition, two methods for measuring functional

cholinergic modulation of dopamine release will be employed. We have not yet performed any studies on the ability of cholinergic compounds to alter release of dopamine in striatal synaptosomes from insecticide-treated mice.

For QNB binding studies, striata and cortex were removed and the tissue processed in a manner similar to that described for GBR 12935 binding. Binding assays were run in triplicate in the absence and presence of unlabelled atropine. To measure total binding, 925 ul of KRH buffer, 25 µl of [^{3}H]QNB solution, and 50 µl of tissue homogenate were mixed and incubated for 60 minutes at 37 °C. The same procedure was followed to measure non-specific binding; however, 875 µl KRH buffer and 50 µl of a 0.2 mM unlabeled atropine solution were substituted for 925 ul of KRH buffer. To stop both reactions, 3 ml cold KRH buffer was added to each tube and the contents were filtered on 25 mm Whatman GF/B filter disks pre-wetted with KRH buffer. Filters were each washed 3 times with 3 ml cold KRH buffer to remove unbound radiolabel. Filters were soaked overnight in 5 ml Scintiverse E before radioactivity was measured. An aliquot (25 μl) of each [^{3}H]QNB solution was mixed with 5 ml Scintiverse E cocktail to calculate the exact [^{3}H]QNB concentrations in each reaction mixture. Nonlinear regression of binding isotherms was used to determine B_{max} and K_d (Prism™, GraphPad Software, San Diego, CA)

Results and Discussion: In previous studies, we found that exposing mice to PM (50 mg/kg) caused an upregulation of muscarinic receptors in both cortex and striatum, as evidenced by an increase in the B_{max} for [^{3}H]QNB binding, whereas there was a significant down regulation by CPF (75 mg/kg) in the striatum (but not cortex) 24 hr after the last treatment. There was little or no effect of any treatment on the K_d value for QNB. Daily systemic injection of pyrethroids (*ca.* 1 mg/kg) in young mice slightly down-regulated cortical expression of muscarinic receptors (5-10%), but this effect was not observed in the striatum (Eriksson and Fredriksson, 1991). Reduction in QNB binding at high doses of CPF, of course, has been previously reported (*e.g.*, Chaudhuri *et al.*, 1993). Moreover, we have also observed a reduction in binding below baseline in at least one additional treatment group (100 mg/kg) with PM (Fig. 16 of last year's report). It therefore appears that muscarinic receptor density is rather precariously regulated or the process is incomplete, at least when evaluated 1 day after the last treatment.

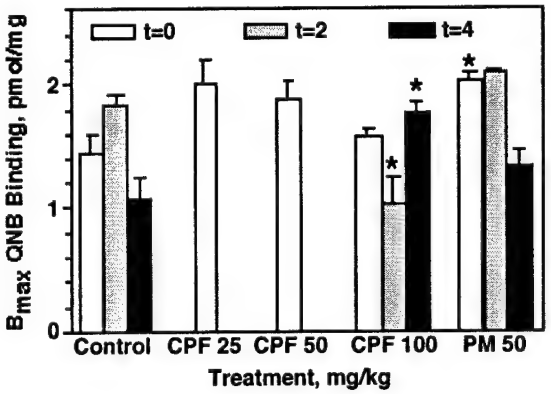


Fig. 11. B_{max} for QNB binding in striatal membranes 1 (t=0), 2 weeks (t=2), and 4 weeks (t=4) after the indicated doses of CPF or PM in striatal membranes (mg/kg). Asterisks indicate treatments significantly different from matched time point controls (T-test, $p < 0.05$).

In the present year, we expanded the QNB studies to include various times after the last treatment to assess reversibility of these effects (up-regulation by 50 mg/kg PM and down-regulation by 100 mg/kg CPF) in both cortex and striatum. In striatal membranes, there was no change in QNB binding by CPF at any of the doses tested 1 day later (Fig. 11). We expected no effect at doses below 100 mg/kg, consistent with our previous studies. The lack of effect 24 hr after the last

treatment of 100 mg/kg may again reflect some variability of effect at this time point. There was, however, a significant decrease in binding in the 100 mg/kg group at t=2 weeks that correlates with the 60% inhibition of cholinesterase activity at t=2 weeks (Fig. 10). This down-regulation had rebounded by t=4 to levels significantly above that of controls. I can find no precedence for this rebound effect in the literature. It may be more a function of the lower control binding we observed at t=4. As we observed previously, 50 mg/kg PM increased QNB binding relative to controls at t=0 (Fig. 11). The up-regulation by PM subsided at later time points.

In the cortex, there was no effect of CPF at any of the doses tested 1 day after the final treatment (Fig. 12). These findings are in accord with our previous studies. Two weeks after the last treatment, binding was strongly down-regulated by CPF, and had recovered to control levels by t=4 weeks. We observed a significant change in QNB binding with PM only at the t=4 time point, and it was a decrease. Thus, we failed to replicate the increased cortical binding we observed previously. More experiments are required to assess the significance of the PM effect at t=4.

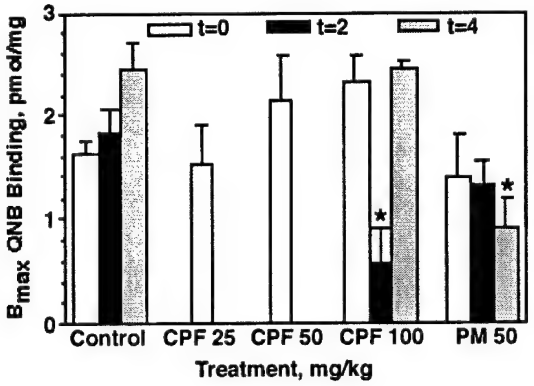
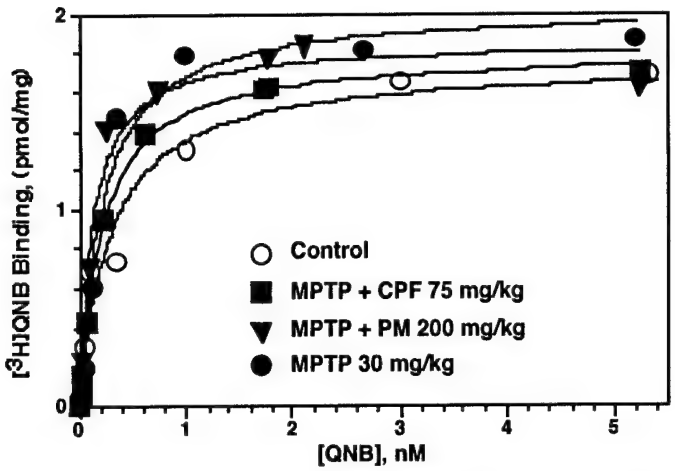


Fig. 12. B_{max} for QNB binding in cortical membranes 1 (t=0), 2 weeks (t=2), and 4 weeks (t=4) after the indicated doses of CPF or PM in striatal membranes. Asterisks indicate treatments significantly different from matched time point control (T-test, $P < 0.05$).

Studies of possible additivity or synergism were also run in mice treated with combinations of neurotoxicants that were assessed 24 hr after the last treatment. Controls showed a B_{max} for QNB binding similar to other groups of mice (Fig. 13). There was no change in the K_d value for QNB binding in any of the treatment groups. A significant up-regulation of QNB binding two weeks after MPTP treatment was observed, consistent with previous findings of Mizukawa *et al.* (1987) using autoradiographic analyses in MPTP-treated mice. When combined with doses of CPF or PM expected to down-regulate or up-regulate binding, respectively, the net effect was a reduction of the MPTP-induced up-regulation. In the case of CPF, the expected down-regulation would appear to be an additive effect. In the case of PM, the underlying cause of the reduced binding is less clear. There are many possible explanations, and we could in the future approach the issue in western blots of choline acetyltransferase levels as a marker of specific cholinergic neurotoxicity. Unfortunately, we have not yet run cortical tissue or the triple treatment group (MPTP+CPF+PM) in QNB binding studies.



Variables	Control	MPTP 30 mg/kg	MPTP + 75 mg/kg CPF	MPTP + 200 mg/kg PM
B_{MAX}	1.740 *a*	2.029 *b*	1.799 *a*	1.852 *a*
K_D	0.2867	0.2101	0.2086	0.1261
Std. Error				
B_{MAX}	0.1075	0.1031	0.03189	0.07828
K_D	0.07498	0.04579	0.01665	0.02695
95% Confid. Limits				
B_{MAX}	1.48 to 2.00	1.79 to 2.27	1.72 to 1.87	1.67 to 2.04
K_D	0.103 to 0.47	0.10 to 0.32	0.17 to 0.25	0.06 to 0.19
Goodness of Fit				
Deg. of Freedom	6	7	7	7
R squared	0.9686	0.9791	0.9971	0.9783
Absolute Sum Sq.	0.09643	0.1188	0.01205	0.1034

Fig. 13. Saturation isotherms of QNB binding in synergism experiments (top), along with nonlinear regression analysis of the data (bottom) from striatal tissue. B_{max} values labeled by different italicized letters are significantly different (ANOVA followed by Student-Newmann-Keuls means separation test).

KEY RESEARCH ACCOMPLISHMENTS

Our most significant observation in the past year was the synergistic interaction between PM, CPF, and MPTP in 28 day analyses of DAT expression. This finding suggests that insecticide exposure could induce or exacerbate idiopathic disease processes, and that it has a slow time course.

Establishment of the NOEL (0.2 mg/kg) for the slow time course of DAT induction was also significant. These findings suggest that the time course of alteration of other biomarkers may be slower than anticipated and that effects could occur at lower doses than previously observed.

We replicated and expanded studies on QNB binding and cholinesterase activity following toxicant exposure to include longer times and combination treatments. No striking effects were observed in studies of combination treatments. Paper to follow.

REPORTABLE OUTCOMES

Meeting Presentations (speaker underlined)

J. S. Gillette and J. R. Bloomquist, Modulation of Murine Striatal Dopamine Transporter Expression by the Pyrethroid Insecticide Permethrin, spring 2002, National Meeting of the Society of Toxicology, Nashville, Tennessee.

Publications

D. Karen, W. Li, P. Harp, J. Gillette, and J. Bloomquist, Striatal Dopaminergic Pathways as a Target for the Insecticides Chlorpyrifos and Permethrin. *NeuroToxicology* 22, 811-817 (2001). This manuscript was "in press" last year. A final copy is included in the appendix of this report.

J. Bloomquist, R. Barlow, J. Gillette, W. Li, and M. Kirby, Selective Effects of Insecticides on Nigrostriatal Dopaminergic Nerve Pathways. *NeuroToxicology* 23, 537-44 (2002). Some data from this project was included in this paper as part of the Proceedings of the "19th International Neurotoxicology Conference: Parkinson's Disease, Environment and Genes." This manuscript was "in review" at the time of last year's report. A final copy is included in the appendix.

J. Gillette and J. Bloomquist, Differential Up-Regulation of Striatal Dopamine Transporter and α-Synuclein by the Pyrethroid Insecticide Permethrin. *Toxicol. Appl. Pharmacol.* (submitted). A final draft copy is included in the appendix of this report.

J. Pittman, C. Dodd, and B. Klein, Immunohistochemical Changes in the Mouse Striatum Induced by the Pyrethroid Insecticide Permethrin. *Intl. J. Toxicol.* (submitted). A final draft copy is included in the appendix of this report.

CONCLUSIONS

Upregulation of the DAT is of greater amplitude, higher potency, and slower time course than previously observed, and through an interaction with α-synuclein (see Gillette and Bloomquist paper in the appendix), may provide a mechanism for pyrethroid-induced neurodegeneration. It has been reported that human α-synuclein has the ability to complex with human DAT, causing acceleration of cellular DA uptake and DA-induced cellular apoptosis (Lee *et al.*, 2001). DA itself can be metabolized to toxic free radical species that may have neurotoxic effects, including inhibition of mitochondrial respiration (Ben-Shachar *et al.*, 1995). Thus, the combined increase in DAT and α-synuclein by pesticides such as PM may exacerbate this potential for cellular toxicity, especially if sufficiently prolonged. Our findings with α-synuclein, while pertinent to these studies, are not part of our plan of work for this project.

The synergistic interaction between PM, CPF, and MPTP in 28 day analyses of DAT expression. suggests that insecticide exposure could induce or exacerbate idiopathic disease processes. It would be useful to assess lower dose, longer term exposures to see if a similar effect occurs.

Down-regulation of muscarinic receptors via QNB binding and cholinesterase activity shows a good correlation between these two biomarkers of cholinergic neurochemistry, especially in longer term exposures. In contrast, toxicant combinations were in some cases antagonistic, yielding little net change in expression. Any correlation of these results with nicotinic receptor regulation will be assessed in epibatidine binding studies.

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APPENDICES

Meeting Abstracts (formatted as per different society guidelines)

For Society of Toxicology, 2002:

MODULATION OF STRIATAL DOPAMINE TRANSPORTER EXPRESSION IN THE C57BL/6 MOUSE BY THE PYRETHROID INSECTICIDE PERMETHRIN.
JS Gillette and JR Bloomquist. Dept. of Entomology, Virginia Polytechnic and State University, Blacksburg, VA, USA.

The pyrethroid insecticide permethrin may play a role in the development of Parkinson's disease as a consequence of Gulf War Syndrome. Previous work in our laboratory has shown that an i.p. dose of 1.5 mg/kg permethrin in the C57BL/6 mouse causes a significant increase in striatal dopamine uptake to 125% of control values. Recently, we have dosed C57 mice with either 0.8 or 1.5 mg/kg permethrin (3 i.p. injections over 2 weeks). To assess reversibility of effects on striatal dopamine transporter (DAT), a portion of the mice were sacrificed on the day following the last dose (t=0), while a second group was sacrificed 2 weeks post last treatment (t=2), and a third group 4 weeks post last treatment (t=4). At t=0, DAT protein as assayed by Western blotting was increased 119% and 107% in the 0.8 and 1.5 mg/kg group, respectively, over that of control mice. DAT binding was analyzed by incubating the ligand [^{3}H]GBR 12935 with striatal synaptosomes from control and treated mice. [^{3}H]GBR 12935 binding was increased 156% and 118% in mice treated with 0.8 and 1.5 mg/kg permethrin, respectively. Preliminary evidence suggests that this effect is persistent, since DAT protein was likewise elevated in the t=2 group of mice. Due to its effects on mouse DAT, the ability of permethrin to synergize the effects of the neurotoxin MPTP was ascertained. MPTP (30 mg/kg) was administered to C57BL/6 mice on day 1 of the treatments, followed the next day by administration of various doses of permethrin from 0.8 mg/kg to 200 mg/kg. Striatal dopamine and its metabolite dihydroxyphenylacetic acid (DOPAC) were determined by HPLC-ECD. Only the highest dose of permethrin (200 mg/kg) in combination with MPTP was able to deplete dopamine significantly greater than MPTP alone. However, DOPAC titers were significantly elevated at doses above 50 mg/kg permethrin, suggesting an increase in dopamine turnover. Taken together, these data suggest that insecticides such as permethrin may have subtle effects on the dopaminergic system, and may be involved in the etiology of Parkinson's disease.

Manuscripts and Papers:

Prepared for *Toxicology and Applied Pharmacology*

Differential Up-Regulation of Striatal Dopamine Transporter and α-Synuclein by the Pyrethroid Insecticide Permethrin

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Key words: Parkinson's disease, striatum, neurotoxicity, pyrethroid

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Abstract

Studies to assess the effects of permethrin on striatal dopaminergic biomarkers gave mice three i.p. doses over a 2 week period, followed by sacrifice 24 hours (t = 0), 2 weeks (t = 2), and 4 weeks after the last treatment (t = 4). At t = 0, dopamine transport was increased at a low dose of permethrin (1.5 mg/kg) and significantly decreased at a high dose (200 mg/kg), compared to controls. Dopamine transporter (DAT) protein as assayed by western blotting was increased 115% in the 0.8 mg/kg group over that of control mice ($P < 0.05$). At t = 2, this value increased to 140% of control, and declined slightly to 133% of control at t = 4 weeks. The mice given the 1.5 mg/kg dose displayed a significant increase in DAT protein only at t = 4, to 145% of controls. Thus, upregulation of the DAT at low doses of PM is variable 24 hr after treatment, and seems to stabilize by t = 4. The threshold dose for increasing DAT expression in western blots by t = 4 was 0.2 mg/kg permethrin. [^{3}H]GBR-12935, used to assay DAT binding, followed the same trend as for the Western blotting data for 0.8 and 1.5 mg/kg doses of permethrin over the 4 weeks post-treatment. At 200 mg/kg permethrin, DAT protein was unchanged vs. controls (t = 0), but had significantly increased by t = 2 and continued to increase at t = 4, suggesting that the reduced dopamine transport at this dose was due to nerve terminal stress and that recovery occurred. The protein α-synuclein was also significantly induced at the 1.5 mg/kg dose at 24 hours post-treatment; however, unlike DAT up-regulation, this effect had declined to control values at t = 2 and t = 4. Maximal induction of α-synuclein protein occurred at a dose of 50 mg/kg permethrin. These data provide evidence that the pyrethroid class of insecticides can modulate the dopaminergic system at low doses, in a persistent manner, which may render neurons more vulnerable to toxicant injury, perhaps leading to Parkinson's Disease.

Introduction

Parkinson's disease (PD), a chronic neurodegenerative disease of unknown etiology, is characterized by a loss of dopaminergic neurons in the substantia nigra, with a resultant depletion of striatal dopamine (DA) (Fearnley and Lees, 1991). In humans, an 80% striatal DA loss is required before overt clinical symptoms appear, after which significant correlation exists between severity of PD symptoms and subsequent DA loss (Hornykiewicz and Kish, 1986).

The etiology of idiopathic PD is thought to be multifactorial (Paganini-Hill, 2001; Siderowf, 2001; Zhang *et al.,* 2000), and it has long been known that there is an epidemiological link between PD and persons who are associated with rural living and agricultural work. In particular, persons exposed to various herbicides and insecticides used in an agricultural setting show an increased risk of developing PD (Semchuck *et al.,* 1992; Semchuck *et al.,* 1993: Gorell *et al.,* 1997). Other work linked exposure to the organochlorine insecticides such as dieldrin to PD (Fleming *et al.,* 1994). The discovery that 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) is able to produce parkinsonism in animals and humans via its oxidative metabolite MPP^+ (Tipton and Singer, 1993), gave further credence to a putative environmental component of PD etiology (Jenner *et al.,* 1992).

The so-called Gulf War Syndrome, which is manifest as various neurological maladies reported by veterans of the Persian Gulf War, may be linked to pesticide exposure. These compounds include pyridostigmine bromide (PB), an anticholinesterase, the insect repellent DEET, and the pyrethroid insecticide permethrin (PM), which was impregnated into the

uniforms of soldiers who served in the war (Hoy *et al.*, 2000). Personnel who served in the Gulf War report a variety of symptoms of neurological dysfunction, with no mortality increases, which have been confirmed by epidemiological studies (Steele, 2001). Further, these symptoms may be related to a spectrum of generalized neurologic injury to the central, peripheral and autonomic nervous systems (Haley *et al.*, 1997a; Haley *et al.*, 1997b). The possibility exists that diseases such as PD may be a long-term consequence of Gulf War Syndrome.

The dopamine transporter (DAT) is a membrane bound carrier molecule that mediates the action of DA in the nerve synapse via the re-uptake of DA into the dopaminergic neuron, and the DAT is also capable of neuronal DA efflux or release (Horn, 1990). In addition, MPP^+ is transported into the neuron via the DAT, where it blocks mitochondrial complex 1 respiration and subsequently causes cell death (Tipton and Singer, 1993). Thus, the DAT is a putative molecular gateway for exogenous and endogenous dopaminergic toxicants, and may be a critical component in the mechanism by which environmental chemicals such as pesticides cause PD (Miller *et al.*, 1999).

Earlier work in our laboratory found that the organochlorine insecticide heptachlor has significant effects on DA transport and causes DAT up-regulation in C57Bl/6 mice at relatively low doses (Kirby *et al.*, 2000; Miller *et al.*, 1999). We also showed that the pyrethroid insecticides deltamethrin and permethrin significantly increase DA uptake in treated mice (Kirby *et al.*, 1999; Karen *et al.*, 2001). The objective of this research was to further characterize the time- and dose-dependence of permethrin's effects on DA transport, transporter ligand binding, and DAT expression. Moreover, because mutations in the α-synuclein gene are associated with some forms of familial PD (Langston *et al.*, 1998; Mizuno *et al.*, 2001), and the herbicide paraquat causes up-regulation and increased aggregation of α-synuclein in C57Bl/6 mice (Manning-Bog *et al.*, 2001), we have expanded our studies to include western blot analysis of α-synuclein expression in PM-treated mice. In this way, we hope to gain insight into the mechanisms by which compounds alter the dopaminergic system and render the neuron more susceptible to toxicant injury.

Materials and Methods

Chemicals

Technical permethrin (mixture of 1-*R,S-cis* and 1-*R,S-trans* isomers) was purchased from Sigma-Aldrich GmbH. Sucrose, HEPES, KCl, and methanol were purchased from Fisher Scientific (Pittsburgh, PA). Methoxytriglycol (MTG), NaCl, $CaCl_2$, $MgSO_4$, and Ponceau-S stain were purchased from Sigma Chemical Co. (St. Louis, MO). GBR 12909 was obtained from Research Biomedicals International (Natick, MA), and [^{3}H]GBR 12935 was obtained from NEN Life Science Products, Inc. (Boston, MA). Acrylamide, bis-acrylamide, ammonium persulfate, N,N,N,N'-tetra-methyl-ethylenediamine (TEMED), and Tris/glycine/SDS buffer were purchased from Bio-Rad (Hercules,CA). ECL Western blotting detection system RPN 2108 and ECL Hyperfilm were purchased from Amersham-Pharmacia Biotech, Buckinghamshire, U.K.

Animals and Treatments

Retired male breeder C57Bl/6 mice (Charles River Labs, Raleigh, NC) were dosed i.p. with permethrin at doses ranging from 0.1 mg/kg to 200 mg/kg. Mice were weighed and

segregated into randomized dosing groups. Doses of permethrin dissolved in MTG were given three times over a two-week period and sacrificed 24 hours after the last dose according to the method of Bloomquist *et al.* (1999). Control mice received MTG vehicle alone. For the time course studies, groups of mice were allowed to live for either two or four weeks post last dosing, at which time they were sacrificed. At the time of necropsy, brain striatal tissues were dissected from the mice and prepared for assay as described below. All procedures were approved by the VPI&SU Animal Care and Use Committee.

Dopamine Uptake Studies.

DA uptake studies were performed according to the method outlined in Kirby *et al.*, (1999), which were based on those of Krueger (1990). Synaptosomes were prepared from fresh striatal tissue dissected from treated mice, and incubated with increasing concentrations of [^{3}H]DA (30 nM - 3 μM) for 2 min. The incubates were washed three times and vacuum filtered, followed by liquid scintillation counting. Uptake rates were determined using parallel incubations with and without sodium ions (equimolar choline chloride substitution) in order to correct for nonspecific transport using the method of Krueger (1990). Aliquots of each synaptosomal preparation were frozen at -20 °C for membrane protein corrections, which was according to the method of Bradford (1976). Uptake parameters (V_{max} and K_m) were determined by nonlinear regression to isotherm plots using Prism™ (GraphPad Software, San Diego, CA).

[^{3}H]GBR 12935 Binding Assay

Striatal tissues were homogenized in ice cold Krebs-Ringer's-HEPES (KRH) buffer (118 mM NaCl, 4.8 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM $MgSO_4$, 20 mM HEPES). Striata were then centrifuged at 18,000 x g, and resuspended for one hour in distilled water. The tissues were again centrifuged at 18,000 x g, resuspended in cold KRH buffer, and stored at -70° C until use. For the assay, samples were thawed and run in duplicate in the absence and presence of 0.5 μM GBR 12909. To measure total binding, [^{3}H]GBR 12935 (100, 50, 25, 12.5, and 6.25 nM) was incubated with KRH buffer and tissue for 2 hours at 4° C. The same procedure was used to measure non-specific binding with the exception that GBR 12909 was added to the reaction mixture. The reactions were stopped by the addition of cold KRH buffer, and the contents of each reaction tube was filtered at 10 psi using 25 mm Whatman GF/B filters pre-soaked in 0.1% BSA. Filters were then washed 3 times with cold KRH buffer to remove unbound radioactivity. The filters were soaked overnight in Scintiverse E, and total counts were measured on a Beckman LS 6500 liquid scintillation counter. Aliquots of [^{3}H]GBR 12935 (100, 50, 25, 12.5, and 6.25 nM) were added to Scintiverse E cocktail to calculate the exact [^{3}H]GBR 12935 concentrations in each reaction mixture. Following protein determination, nonlinear regression to isotherm plots was used to determine B_{max} and K_d using Prism™ (GraphPad Software, San Diego, CA).

Western Blot Analysis

Striatal synaptosomes from C57 BL/6 mice were homogenized in cold isolation buffer and centrifuged at 1000 x g for 15 min. The supernatant was removed and centrifuged at 10,000 x g for 15 min. The resulting pellets were resuspended in a small volume of KRH buffer and stored at -70° C until use. At the time of assay, the tissues were thawed, mixed with sample buffer (60

mM Tris, pH 6.8, 2% SDS, 100mM DTT, 0.001% bromphenol blue), and heated at 80° C for 5 min. Samples were run on either a 10% SDS gel (DAT, synaptophysin), or a 12% SDS gel (α-synuclein) and transferred to a nitrocellulose membrane according to the method of Towbin *et al.* (1979). For the DAT, α-synuclein, and synaptophysin SDS gels, 10, 5, and 2 µl of protein were loaded, respectively. After transfer was complete, the membrane proteins were stained with Ponceau S dye to verify transfer. The membrane was incubated overnight in 5% dry milk at 5° C, followed by an overnight incubation at 5° C in either a mouse monoclonal anti-α-synuclein (Biodesign International, Saco, ME), a rat monoclonal anti-DAT, or a rat monoclonal anti-synaptophysin primary antibody (Chemicon Int'l, Temecula, CA). The blot was washed with TBST buffer and incubated in an appropriate peroxidase-linked secondary antibody (Sigma Chemical Co.) for one hour at room temperature. After a second washing with TBST buffer, the blot was developed using the ECL Amersham Chemiluminescence kit (Amersham-Pharmacia Corp.), and exposed to ECL hyperfilm (Amersham-Pharmacia Corp.) for varying lengths of time. Protein bands on the film were quantitated using the Kodak EDAS 290 system (Eastman Kodak Co.). Protein content was determined by the method of Bradford (1976).

Statistical Analysis

Statistical analysis was determined using one-way ANOVA and Student-Newman-Keuls means separation if a statistically significant effect of treatment was observed. Other statistical comparisons were by T-test calculations. Analysis was performed using InStat™ (GraphPad Software, San Diego, CA).

Results

Permethrin given over a wide range of doses was shown to maximally increase DA uptake in striatal synaptosomes at a dose of 1.5 mg/kg, to 134% of control value (Table 1). At higher doses, PM has the opposite effect in that DA uptake was significantly depressed to 50% of control at a 200 mg/kg dose (Table 1). Since maximal DA uptake occurred at 1.5 mg/kg permethrin, it was originally decided to select the PM doses of 0.8 and 1.5 mg/kg for the initial [^{3}H]GBR 12935 binding assays and western blotting studies.

At 24 hours posttreatment, [^{3}H]GBR 12935 binding was significantly elevated in the 0.8 mg/kg dose group to 137% of control, but was unchanged in the 1.5 mg/kg dose group (Fig. 1). Other groups of mice from the same experimental cohort were assessed on either 14 or 28 days beyond the last dose, with no further treatments given. At t = 14 days, GBR 12935 binding increased to 157% of control in the 0.8 mg/kg dose group, with the 1.5 mg/kg dose group remaining unchanged. At t = 28 days, both the 0.8 and 1.5 mg/kg dose groups demonstrated significantly increased [^{3}H]GBR 12935 binding (~ 130% for both groups) over that of controls (Fig 1). [^{3}H]GBR 12935 binding at 24 hours was not significantly different from that of controls at PM doses of 100 mg/kg and 200 mg/kg; however, a dose dependent trend for a decrease at both doses was evident (Fig. 2).

Western blotting of DAT protein largely reflected the above [^{3}H]GBR 12935 binding results for each time period tested. At t = 24 hrs, 14 days, and 28 days post-treatment, DAT protein was significantly elevated to 115%, 140%, and 133% of controls respectively, in the 0.8

mg/kg treatment group (Fig. 3). The 1.5 mg/kg treatment group did not show a significant induction of DAT protein until the t = 28 day time period, to 145% of control (Fig. 3). A similar effect on DAT was observed in mice treated with 200 mg/kg PM (Fig. 4). As before, DAT protein was not significantly different in the control vs. treatment groups 24 hours post-treatment; however at t = 14 days and t = 28 days, DAT protein was significantly upregulated to 135% and 147% of control, respectively (Fig. 4).

In an effort to determine the threshold dose of PM for up-regulating DAT protein expression, doses of 0.1 mg/kg, 0.2 mg/kg, or 0.4 mg kg PM were administered according to the standard dosing scheme and sacrificed at t = 24 hrs, 14 days, or 28 days post treatment. At 24 hours after the last treatment, there was a significant increase in DAT protein at doses of 0.2 and 0.4 mg/kg, but not at 0.1 mg/kg. This effect on DAT protein was persistent for both doses at both 2 and 4 weeks. Again, in the highest dose group, there was a significant increase in DAT protein from t = 24 hrs to t = 14 days, which the remained constant until t = 28 days. Thus, the threshold dose for significant up-regulation of DAT protein by technical PM was 0.2 mg/kg (Fig. 5).

Alpha-synuclein protein was also quantified by western blotting in the same 0.8 mg/kg and 1.5 mg/kg dose groups used to assay DAT (Fig. 6). At a PM dose of 1.5 mg/kg, α-synuclein was upregulated in the mice sacrificed 24 hrs after last PM dose to 180% of control. However, unlike DAT expression, this effect was not persistent as evidenced by the lack of response in both the 14 and 28-day mice (Fig. 6). Alpha-synuclein protein expression demonstrated a bell-shaped curve over a wide range of PM doses from 25 mg/kg to 200 mg/kg, with a peak increase at 50 mg/kg to 244% of control. Alpha-synuclein protein was significantly up-regulated at doses of 25 and 100 mg/kg PM (145% and 137% of controls, respectively), and was not significantly changed at the 200 mg/kg PM dose (Fig. 7).

Synaptophysin, a neuronal vesicle-bound protein, was used in western blotting techniques to quantify synapses in the above tissues. It was also used to verify uniform loading of protein in western blots. Synaptophysin protein remained unchanged across treatment groups in all tissues tested, as opposed to the up-regulation observed for DAT and α-synuclein (Fig. 8). Uniform protein loading was also ensured by semi-quantitatively measuring the Ponceau-S staining of each western blot lane, which again remained constant across all treatment groups and time points.

Discussion

The present study confirms and extends the results of Karen et al. (2001), and establishes a potent, slowly developing up-regulation of the DAT by the pyrethroid, permethrin. In general, pyrethroids are quickly metabolized and excreted from the body, although some pyrethroid metabolites may be persistent (Casida *et al.*, 1983). In light of this rapid metabolism then, it was surprising to us that at doses as low as 0.2 mg/kg PM, DAT protein was significantly induced at 4 weeks post-treatment. Thus, DAT expression can occur at doses which are about an order of magnitude below that which have been previously reported to have this effect (Karen *et al.*, 2001). It is also noteworthy that of the four PM stereoisomers, only one of them (1*R-cis* configuration across the cyclopropane ring) has significant acute toxicity to mammals (Casida *et al.*, 1983). If this isomer is also solely responsible for the observed effects on the DAT, then the

actual doses of PM responsible for this effect are 4-fold lower than those used in this study. Support for this conclusion is found in the studies with deltamethrin. This compound is composed of a single 1*R,cis*,α*S* isomer that is toxic to mammals (murine ip LD_{50} = 10 mg/kg, Casida *et al.*, 1983) and it up-regulates the DAT (Kirby *et al.*, 1999).

It is compelling that our published DA uptake data, in which transport is increased at low PM doses (Karen *et al.*, 2001), is mirrored by the [^{3}H]GBR 12935 binding studies and quantitation of DAT protein by western blotting of this study. There has been some debate over the use of [^{3}H]GBR 12935 as a DAT-specific ligand. Pristupa *et al.* (1994) found that binding of [^{3}H]GBR 12935 in human COS-7 cells transfected with human DAT was not saturable up to 22 nM. It has also been shown that [^{3}H]GBR 12935 is able to bind to dopamine-insensitive "piperazine acceptor sites", one of which has been identified as human CYP2D6 (Allard *et al.*, 1994; Hiroi *et al.*, 1997). In our experiments, [^{3}H]GBR 12935 binding was specific and saturable, showed excellent correlation with other biomarkers as mentioned above, and yielded a B_{max} value for C57Bl/6 control mice of about 3 pmol/mg protein, similar to values reported by Horn (1990) for rat striatum (5.5 pmol/mg) or human caudate tissue (2.7 pmol/mg).

The lack of a statistically significant response at the 1.5 mg/kg dose until week 4 in the binding and western blot studies was surprising, since the maximal increase in DA uptake occurred at this dose 24 hours post-treatment. It is likely that there are cohort-related differences in the effectiveness of the 1.5 mg/kg PM dose. This possibility is supported to some extent by our previous data in which up-regulation of DAT protein did occur at the 1.5 mg/kg dose at 24 hours post treatment (Bloomquist *et al.*, 2002). A slowly-developing effect on DAT was observed across all doses tested, since DAT protein was induced to a greater extent at 2 and 4 weeks vs. 24 hours post treatment in mice given 0.2-0.4 mg/kg (Fig. 5) and 200 mg/kg PM (Fig. 4). This trend is even evident at t = 4 weeks for the 0.1 mg/kg dose (Fig. 5), indicating that induction of DAT up-regulation is variable or delayed until 28 days post treatment, when it stabilizes. The slow time course of this effect suggests that longer term studies should be undertaken. At high doses of PM (e.g., 200 mg/kg), the delayed response in DAT up-regulation may be due to nerve terminal toxicity caused by the high PM dose, since this dose causes a reduction in mitochondrial function in similar studies (Karen *et al.*, 2001). After a recovery period, DAT up-regulation again becomes evident. Persistent DAT up-regulation was also noted in Sprague-Dawley rats exposed to heptachlor in the gestational, perinatal, and adolescent stages of development. In this case, DAT binding to [^{3}H]-mazindol remained significantly increased into adulthood (Purkerson-Parker *et al.*, 2001).

Unlike DAT, the up-regulation of α-synuclein protein was not a persistent effect, and was not present in the two and 4-week post treatment groups. Mutations in α-synuclein are believed to cause the fibrillar aggregates that are the major components of Lewy bodies, a pathological hallmark of PD (Murray *et al.*, 2001). Mutations in the α-synuclein gene have also been associated with some forms of familial PD (Langston *et al.*, 1998; Mizuno *et al.*, 2001). Manning-Bog *et al.* (2001) found the herbicide paraquat to cause up-regulation and increased aggregation of α-synuclein in C57Bl/6 mice. This up-regulation was also not persistent, with protein levels returning to control values by post-treatment day 7.

It has been reported that human α-synuclein has the ability to complex with human DAT, causing acceleration of cellular DA uptake and DA-induced cellular apoptosis (Lee *et al.*, 2001). DA itself can be metabolized to toxic free radical species that may have neurotoxic effects, including inhibition of mitochondrial respiration (Ben-Shachar *et al.*, 1995). Thus, the combined increase in DAT and α-synuclein by pesticides such as PM may exacerbate this potential for cellular toxicity, especially if sufficiently prolonged. Our observed up-regulation of α-synuclein may also be a compensatory mechanism, since it has been reported that α-synuclein is able to inactivate c-Jun N-terminal kinase (JNK), thus protecting cells against oxidative stress (Hashimoto *et al.*, 2002). Although the signalling pathway for protein up-regulation by pyrethroids is unknown, these compounds have a high affinity interaction with the β-subunit of GTP binding proteins (Rossingnol, 1991 a,b), and increase protein phosphorylation levels by enhancing the effects of either protein kinase C (Enan and Matsumura, 1992) or protein kinase A (Matsumura *et al.*, 1989).. The bell-shaped dose-response curves observed for all proteins suggest that the signalling pathways are modified at different threshold doses, and that the pathway apparently shuts down at higher doses, at least at 24 hours.

Our data therefore, demonstrate that permethrin, in addition to heptachlor and deltamethrin, is able to up-regulate DAT protein at doses far below those previously reported. Moreover, we have shown this to be a slowly developing and persistent effect, which is evident long after the administered PM has ostensibly been metabolized. If DAT is indeed a gateway for entry of toxicants into the neuron, then persistent DAT up-regulation may render the cell highly vulnerable to toxicant injury. The effects of PM on DAT and α-synuclein are more significant in light of the fact that PM absorption is modulated by the co-administration of PB and DEET, as used in the Gulf War (Baynes *et al.*, 2002). We thus have a possible mechanism whereby persons reporting symptoms of Gulf War may be predisposed to neurotoxicant injury and the subsequent development of PD.

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TABLES

Table 1. Dopamine uptake in C57Bl/6 striatal synaptosomes from PM-treated mice. Values are means ± SEM and *denotes significant difference from control (Students t-test, $P < 0.05$). these data are the basis for the present study and are taken from Karen *et al.* (2001).

Treatment (mg/kg)	DA Uptake (pmol/min/mg)
Control	165 ± 13
0.8	178 ± 10
1.5	219 ± 17*
6	181 ± 16
100	125 ± 43
200	72 ± 14*

Figures

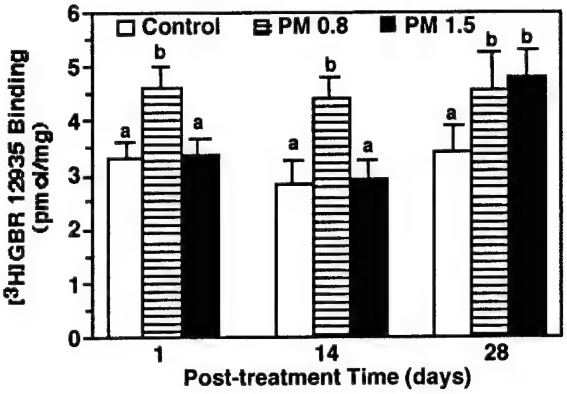

Fig. 1. Time course experiments of [^{3}H]GBR 12935 binding in C57Bl/6 mice treated with 0.8 mg/kg or 1.5 mg/kg permethrin. Bars represent means with SEM in this and all subsequent figures. Letters denote results of ANOVA with Student-Newman-Keuls means separation test, where treatments within a particular time period (1, 14, or 28 days) are significantly different ($p<0.05$) when labeled by different letters.

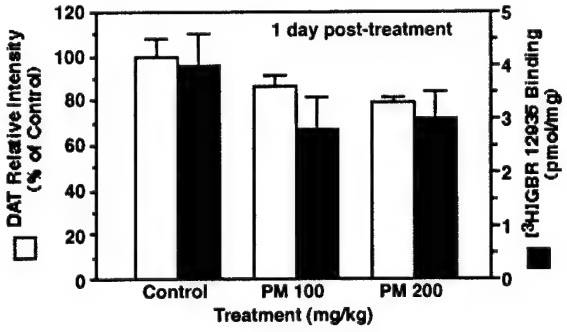

Fig. 2. DAT western blot densitometry and [^{3}H]GBR 12935 binding in C57Bl/6 mice sacrificed 1 day after the final treatment with 100 or 200 mg/kg permethrin. No statistically significant differences were observed (T-test, $p>0.05$).

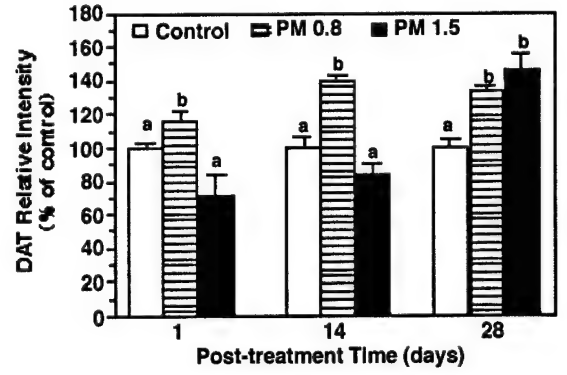

Fig. 3. Time course experiments of DAT western blot densitometry in C57Bl/6 mice treated with 0.8 mg/kg or 1.5 mg/kg permethrin. ANOVA was performed on the raw data before calculation as % of control. ANOVA and means separation as described for Fig. 1.

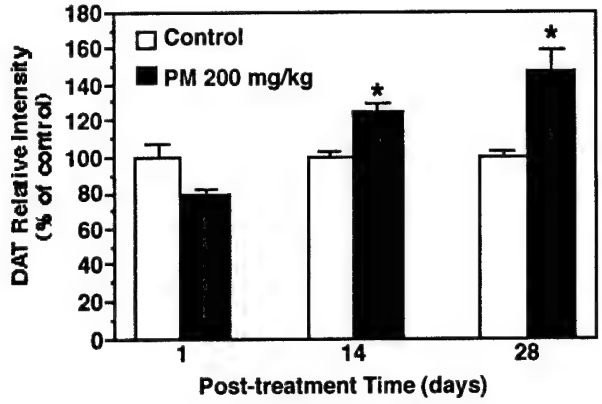

Fig. 4. Time course studies using densitometry of DAT western blots of striatal synaptosomes from mice treated with 200 mg/kg PM. Raw data were analyzed by T-test before calculation as % of control, and bars labeled by an asterisk for a particular time point (1, 14, or 28 days) are significantly different ($p<0.05$).

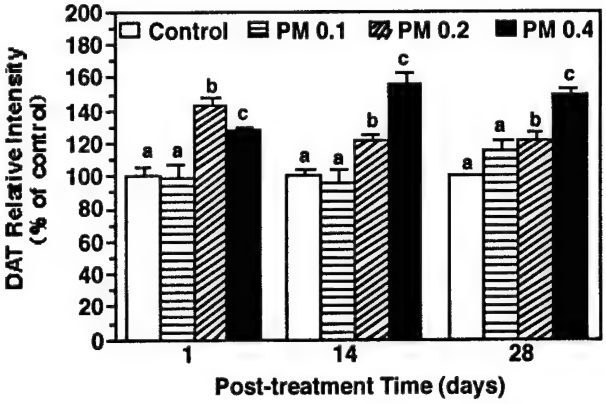

Fig. 5. Time course studies using densitometry of striatal synaptosomes on DAT western blots from mice treated with either 0.1, 0.2, or 0.4 mg/kg PM. ANOVA and means separation as described for Fig. 3.

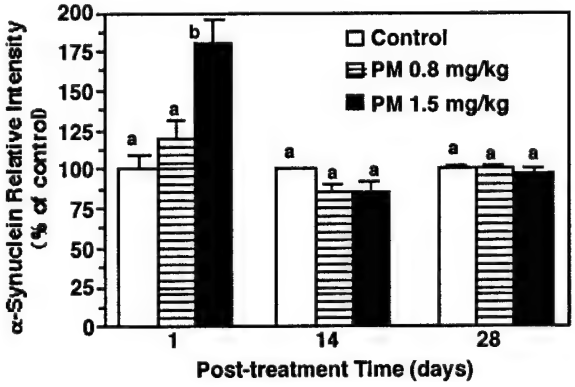

Fig 6. Densitometry of α-synuclein in western blots of striatal synaptosomes from mice treated with either 0.8 or 1.5 mg/kg permethrin at 1, 14, and 28 days post-treatment. ANOVA and means separation as described for Fig. 3.

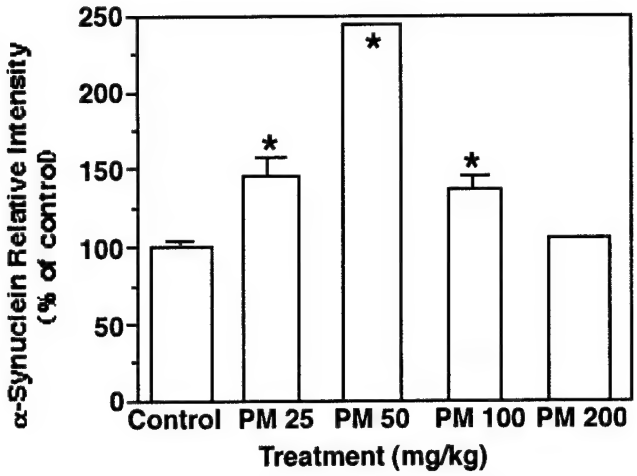

Fig. 7. Densitometry of α-synuclein in western blots of striatal synaptosomes from mice treated with increasing doses of permethrin. Letters denote results of a T-test, where bars labeled by an asterisk for a particular time point (1, 14, or 28 days) are significantly different from controls ($p<0.05$). The SEM of the 50 mg/kg group was too small to be seen at the y-axis scaling of the graph.

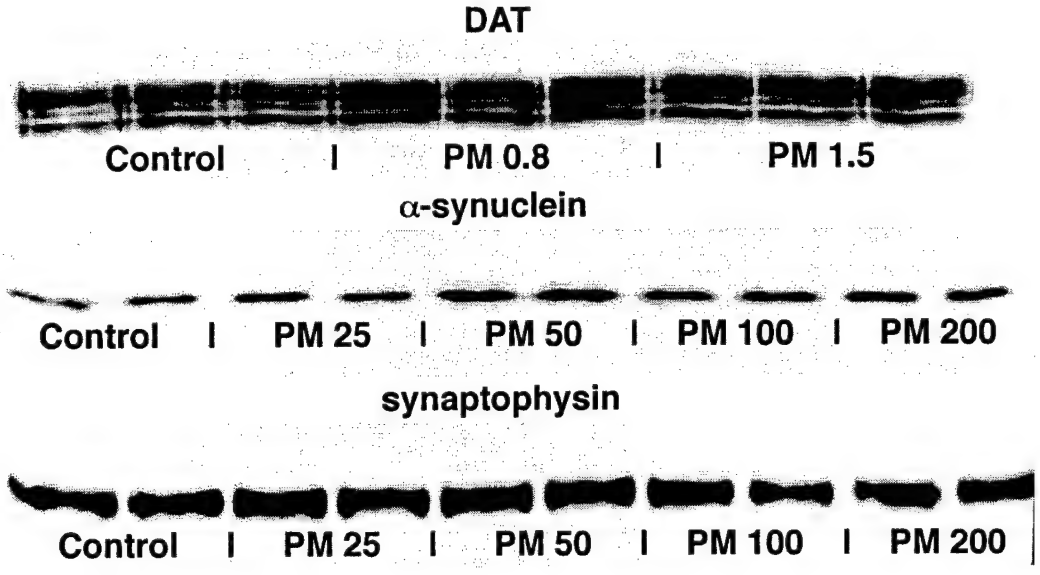

Fig. 8. Examples of representative western blots of DAT, α-synuclein, and synaptophysin. Treatments are expressed below stained bands, and are mg/kg PM.

For: *International Journal of Toxicology*

Immunohistochemical Changes in the Mouse Striatum Induced by the Pyrethroid Insecticide Permethrin

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Running head: Permethrin and mouse striatum

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ABSTRACT

Epidemiological studies have linked insecticide exposure and Parkinson's disease. In addition, some insecticides produce damage or physiological disruption within the dopaminergic nigrostriatal pathway of non-humans. This study employed immunohistochemical analysis in striatum of the C57BL/6 mouse to clarify tissue changes suggested by previous pharmacological studies of the pyrethroid insecticide permethrin. Dopamine transporter, tyrosine hydroxylase and glial fibrillary acidic protein immunoreactivities were examined in caudate-putamen to distinguish changes in amount of dopamine transporter immunoreactive protein from degeneration or other damage to dopaminergic neuropil. Weight-matched pairs of pesticide-treated and vehicle control mice were dosed and sacrificed on the same days. 0.8, 1.5 and 3.0 mg/kg of permethrin were the low doses and 200 mg/kg the high dose. Brains from matched pairs of mice were processed on the same slides using the avidin-biotin technique. Four fields were morphometrically located in each of the serial sections of caudate-putamen, digitally photographed and immunopositive image pixels were counted and compared between members of matched pairs of permethrin-treated and vehicle control mice. For low doses, only 3.0 mg/kg produced a significant decrease in dopamine transporter immunostaining. The high dose of permethrin did not produce a significant change in dopamine transporter or tyrosine hydroxylase immunostaining, but resulted in a significant increase in glial fibrillary acidic protein immunostaining. These data suggest that a low dose of permethrin can reduce the amount of dopamine transporter immunoreactive protein in the caudate-putamen. They also suggest that previously reported reductions in dopamine uptake of striatal synaptosomes of high dose mice may be due to non-degenerative tissue damage within this region as opposed to reductions of dopamine transporter protein or death of nigrostriatal terminals. These data provide further evidence that insecticides can affect the primary neurodegenerative substrate of Parkinson's disease.

Keywords Permethrin, Striatum, Dopamine Transporter, Tyrosine Hydroxylase, Glial Fibrillary Acidic Protein, Parkinson's Disease

INTRODUCTION

Despite a wealth of investigation into Parkinson's disease (PD), the etiology of the disorder remains elusive. Although there is agreement on a significant genetic contribution in some forms of early onset Parkinson's-like disorders, the role of genetic inheritance in the most common form of the disease, adult or late onset PD, remains equivocal (Olanow & Tatton, 1999; Tanner et al., 1999; Sveinbjornsdottir et al., 2000; Mouradian, 2002). This, in addition to the fact that the xenobiotic compound 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) is used to produce one of the principal mammalian models for Parkinson's disease (Royland & Langston, 1998; Schmidt and Ferger, 2001), has prompted a great deal of work on potential environmental causes or triggers for PD.

Insecticides are a widely used class of environmental chemicals that induce their toxic effects by acting on the nervous system. A number of epidemiological studies support a link between insecticide exposure and Parkinson's disease in humans (Semchuk et al., 1992; Butterfield et al., 1993; Gorell et al., 1998; Corrigan et al., 2000; Zorzon et al., 2002). Furthermore, a number of recent experimental studies have shown that some insecticides can produce damage or physiological disruption within the dopaminergic nigrostriatal pathway (Bloomquist et al., 1999; Miller et al., 1999; Betarbet et al., 2000; Karen et al., 2001; Purkerson-Parker et al., 2001; Kirby et al., 2001, 2002). This pathway is the principal focus of degeneration in idiopathic PD (DiMonte and Langston, 1995; Poewe and Wenning, 1998).

The synthetic pyrethroid insecticides are derivatives of the natural insecticidal pyrethrins found in the chrysanthemum. The principal target of the pyrethroids, in both insects and mammals, is the voltage-gated sodium channel (Ghiasuddin and Soderlund, 1985; Ray, 2001). The opened state of the channel is prolonged, which produces a hyperexcitablity within the target tissue. There is some

evidence that other sites in excitable tissues may be affected as well, such as voltage-gated calcium channels (Hagiwara et al., 1988; Duce et al., 1999), γ-aminobutyric acid receptors (Lawrence et al., 1985; Bloomquist et al., 1986) and neurotransmitter membrane transporters (Kirby et al., 1999; Karen et al., 2001; Bloomquist et al., 2002).

Pyrethroids have been reported to affect the function of components of the dopaminergic nigrostriatal pathway, sometimes in a selective fashion. For example, in rats, decamethrin produced effects on electroencephalographic recordings in striatum and substantia nigra prior to affecting other brain regions (Ray, 1980). In addition, deltamethrin-induced increases in blood flow were greater in caudate nucleus compared with cerebral cortex (Ray, 1982). In rabbit brain slices, fenvalerate was capable of inducing the release of dopamine and acetylcholine from striatum, but did not cause release of norepinephrine or acetylcholine from hippocampus (Eels and Dubocovich, 1988). In neurotransmitter release studies using pre-loaded synaptosomes, it has been shown that deltamethrin induces dopamine release from striatal synaptosomes at EC_{50} values 2.4-8.6 more potent than cortical synaptosomes containing serotonin or glutamate (Kirby et al., 1999; Bloomquist et al., 2002). Pyrethroids have also been shown to increase the concentration of the dopamine metabolite 3,4-dihydroxyphenylacetic acid (DOPAC) in the striatum (Doherty et al., 1988). In prenatally exposed rat pups examined as adults, a fenvalerate-induced increase in DOPAC and DOPAC/dopamine ratio suggested an enhanced activity of the dopaminergic nigrostriatal pathway that was not seen for noradrenergic or serotonergic systems (Lazarini et al., 2001).

Pyrethroid insecticides have also been shown to modulate neurotransmitter uptake (Kirby et al., 1999; Karen et al., 2001; Bloomquist et al., 2002). A recent study has demonstrated that the type I pyrethroid permethrin can alter maximal dopamine uptake (V_{max}) by mouse striatal synaptosomes (Karen et al., 2001) . At low doses V_{max} was increased, while at higher doses V_{max} was decreased, as was open field behavior, compared to vehicle controls. The lack of an accompanying change in K_m suggested that these pharmacological changes reflected an alteration in the amount of dopamine transporter (DAT) present within the synaptosomal tissue sample, rather than a change in the efficiency of the transporter. Furthermore, the reversal of increased maximal dopamine uptake seen at the high doses of permethrin could reflect damage or degeneration of dopaminergic neuropil within the striatum. In the present study, immunohistochemical techniques were employed in the mouse striatum to clarify the tissue changes suggested by the aforementioned kinetic studies. This technique permitted the evaluation of changes in a topographically defined region of the striatum; that which contains the highest density of nigrostriatal dopaminergic afferents (Heimer et al., 1995) and the region most likely to be affected in PD (Kish et al., 1988; Graybiel et al., 1990; Miller et al., 1999). DAT, tyrosine hydroxylase (TH) and glial fibrillary acidic protein (GFAP) immunoreactivities were examined in an attempt to distinguish changes in the amount of the DAT from degeneration or other damage to dopaminergic striatal neuropil.

MATERIALS AND METHODS

Animals and Treatments

Male C57BL/6 retired breeder mice, 7-9 mos. old at the time of the experiment, were obtained from Harlan Sprague-Dawley, Dublin, VA, USA. Mice were assigned to groups by weight, such that every mouse designated for insecticide treatment had a paired, weight-matched control. These weight-matched pairs were randomly assigned to different dosage groups. Insecticide-treated mice were given i.p. injections of permethrin (Sigma Chemical Co., St. Louis, MO, USA) in a methoxytriglycol vehicle, while control mice received vehicle alone. Regardless of dose, all mice received 3 injections over a two week period according to the methods of Bloomquist et al. (1999) and Karen et al. (2001), with Day 1 being the first injection, followed by injections on Days 8 and 15 and sacrifice on Day 16. For every permethrin-treated mouse that was sacrificed on a given day, its weight-matched, vehicle control partner was also sacrificed. Mice receiving 0.8, 1.5 or 3.0 mg/kg of permethrin were considered the "low dose" group and those receiving 200 mg/kg were

considered the "high dose" group. These doses were selected since permethrin treatments of similar magnitude were previously shown to produce the greatest changes in dopamine uptake in the mouse striatal synaptosome preparation (see Karen et al., 2001). In addition, the extent of the dosing spectrum was limited to these values in order to maximize resources and minimize the number of mice used in the experiment.

Fixation, Tissue Sectioning and Histochemistry

On the day of sacrifice, the permethrin-treated and matched vehicle control mice were deeply anesthetized with sodium pentobarbital and perfused transcardially with a phosphate buffered saline (PBS) rinse (0.05M, pH 7.4), followed by 4% paraformaldehyde fixative solutions (in 0.1M phosphate buffer) at respective pH values of 7.0 and 10.5. Brains were then removed and post-fixed in the pH 10.5 fixative for 4 hr., rinsed 3 times with PBS and cryoprotected overnight in 10% sucrose at 4°C.

Using a cryostat, 16 μm sections through the striatum were cut onto slides, beginning at the rostral-most appearance of the lateral ventricle. For the high dose mice, three consecutive sections were cut to provide exposure to tyrosine hydroxylase (TH) antibody (Protos Biotech Corp., New York, NY, USA, #CA-101), cresyl violet staining and either DAT antibody or GFAP antibody (DAKO Corp., Glostrup, Denmark, #Z0334). For the low dose mice, two consecutive 16 μm sections were cut, respectively, for exposure to DAT antibody (Chemicon Int., Temecula, CA, USA, #AB1591P) or cresyl violet staining. Sections for TH and GFAP staining were not prepared for this condition since previous work provided no reason to suspect death or damage of striatal neuropil following the low dose regimen (see Karen et al., 2001). The sectioning sequence was repeated at 320 μm intervals, through the striatum, until 12 sets of multiple sections were cut. One of these sets included an additional slide to be used as an omission control for non-specific staining. After completing the sectioning of a permethrin-treated brain, corresponding sections from the matched vehicle brain were placed on the same slide, which insured identical histochemical treatment for both sections. The two sectioned brains comprised a single case. The slide positions of the treated and vehicle control brains were counterbalanced across cases.

Immunohistochemistry was performed directly on the slides according to a modification of methods previously outlined by this laboratory (Klein & Blaker, 1990; Klein et al., 1992; Misra & Klein, 1995). Briefly, sections were rinsed with PBS, incubated with 3% hydrogen peroxide to quench endogenous peroxidase and rinsed again. The slides were then incubated sequentially in (1) 10% normal goat blocking serum containing 0.15% Trinton-X-100 and (2) polyclonal rabbit antisera to either DAT (1:600), TH (1:400) or GFAP (1:6400), overnight, at room temperature. Tissue was then processed by the avidin-biotin complex method using a Vectastain Elite kit (Vector Labs, Burlingame, CA, USA, #PK6101) and 0.05% diaminobenzidine in 0.01% hydrogen peroxide as the chromogen. Sections were then dehydrated in alcohols, cleared in xylene and coverslipped. Alternate sections were stained with 0.3% cresyl violet in 50% ethanol. Diluent for primary and secondary (biotinylated goat anti-rabbit, included in the kit) antibody solutions was 1% goat serum in PBS containing 0.15% Trinton-X-100. Tissue incubations were performed in a humidity chamber. Striatal immunostaining produced with the three primary antibodies can be seen in Figures 1 and 2. The photographs are simply intended to show examples of the general quality of tissue staining obtained in the study. The detection of differences in the amount of labeled neuropil, between vehicle and insecticide-treated tissue, required the quantitative analysis of digital images described below.

Morphometeric Location of Fields for Image Analysis

An individual data point in this study was defined as the difference in the amount of immunoreactive neuropil (DAT, TH or GFAP) between a brain section from a permethrin-treated mouse and the corresponding section from its matched vehicle control, located on the same slide. The amount of immunoreactive neuropil in a given brain section was a mean, calculated from four

3070 μm^2 fields, distributed within the dorsolateral portion of the striatum (Figure 1B). This region is the primary striatal target of dopaminergic neurons originating from the rodent substantia nigra pars compacta (Heimer et al., 1995). In order to insure consistent sampling between sections from treated and matched vehicle control sections, a morphometric procedure was used to define the location of the four fields within each brain section. Consistent identification of the caudate-putamen, across sections, was aided by the fact that, 1) throughout most of it's rostrocaudal course, the caudate-putamen is bordered dorsally, ventrally and laterally by the external capsule, a well-defined band of white matter, 2) the dorsomedial border of the nucleus is adjacent to and can be easily distinguished from the cavity of the lateral ventricle and, 3) the ventromedial border of the nucleus can be histologically distinguished from the less densely staining globus pallidus (see Figure 1A) in both immunostained and cresyl violet stained sections (Franklin & Paxinos, 1996). In the rostral third of the nucleus, where the external capsule does not extend to the ventral border, the lateral striatal stripe and the anterior commissure were used to locate the ventral border of the caudate-putamen and distinguish it from the nucleus accumbens.

Morphometric location of the four analysis fields was done on camera-lucida tracings of brain sections. Initially, a central point of the caudate-putamen was operationally defined as follows: the dorsal and ventral tips of the nucleus were located with the aid of the external capsule. At the dorsoventral midpoint of the nucleus, a horizontal line was drawn extending from the external capsule laterally, to the pallidal border medially. The midpoint of this line was operationally defined as the center point of the caudate putamen.

After operationally locating the center point of the caudate–putamen, the outer circumference of the nucleus was traced from the ventral-most tip to the dorsal border with the lateral ventricle. Along this arc, marks were made at 1/3 and 2/3 of the total distance. Lines were then drawn from these marks to the center point of the nucleus. Along each of these two radii, marks were made at 1/4 and 1/2 the distance between the perimeter of the nucleus and the center point. This yielded four marks, designating the centers of the four fields that were to be digitally photographed for image analysis (Figure 1B). Around these fields, numerous tissue landmarks were traced, such as unstained fiber bundles or imperfections, to aid in relocating these fields for photography. The fields identified by the procedure described above were consistently distributed within the dorsolateral quadrant of the caudate-putamen and permitted measurements from similar regions within permethrin-treated and matched vehicle control brains.

As alluded to above, and as can be seen in Figs. 1 and 2, coronal sections through the striatum are characterized by fairly numerous, unstained fiber bundles of passage, the pattern of which varies among individual animals. In order to minimize the effect of these fibers of passage upon the analysis, when such a bundle fell within the measurement field designated for photography, the position of the field was adjusted to incorporate the closest adjacent field of homogenous immunolabeling. Thus, the fields such as those depicted in Fig. 1 could vary by as much as 50 μm in any direction, but all still remained within the dorsolateral quadrant of the caudate-putamen.

Image Analysis

The amount of immunostained neuropil in photographs of each of the four fields within a section was quantified using the "Histogram" feature of Abobe Photoshop 5.0 (Adobe Systems, Inc., San Jose, CA, USA). Digital images of a given size are comprised of a fixed array of loci called pixels. For any selected area of pixels from a digital photograph, the "Histogram" function can determine the mean grayscale value (0-255, where 0 is black and 255 is white) and the number of pixels that are darker than a specified grayscale value.

A threshold value for immunostained neuropil was determined by sampling the mean grayscale value of varicosities, that were visually judged to be unambiguously immunopositive, within every brain section of the vehicle control mice. The grayscale value for two such varicosities was

averaged for each field and the mean across the four fields was taken as the average value for the section. The mean across all sections from each vehicle control mouse was determined, and the mean grayscale value across all vehicle control mice was used as the threshold value for identifying immunostained neuropil in both vehicle control mice and permethrin-treated mice. Image pixels as dark, or darker than this value, were then considered as immunopositive labeling, irrespective of how much darker they were. Pixels lighter than this threshold value were not counted. Using the above-noted threshold value, the number of pixels darker than threshold was counted for each photograph of the four measurement fields from each striatal section. This value was averaged across the four measurement fields from each section and was considered the amount of immunostained neuropil in that section. These immunopositive pixel counts were then compared between vehicle and insecticide-treated mice.

Statistical Analysis

As alluded to above, on each microscope slide, a brain section from a permethrin-treated animal was paired with a section from a matched vehicle control animal. These mice were injected on the same days, sacrificed on the same day and the sections were taken from similar rostrocaudal positions within the striatum. In addition, the paired sections on a slide shared identical tissue processing conditions. Therefore, the individual data points subjected to statistical analysis in this study were the differences in immunopositive staining (pixel counts), as described above, between the paired permethrin-treated and matched vehicle control sections on a microscope slide.

Initially, scatterplots were used to assess the effects of microscope slide section position and date of tissue processing on differences in immunostained neuropil between paired sections. Based on this analysis, an ANOVA model, fitted using the GLM procedure of SAS (SAS Institute Inc., Cary, NC, USA), was used to correct for the effect of processing date on each data point. The corrected differences in immunostaining were then consistent with the assumptions for analysis by a paired t-test. For a pair of permethrin-treated and matched vehicle control brains, the difference in immunostained neuropil was averaged across microscope slides. Then, for each dose concentration group, the grand mean of these corrected mean differences was tested for its difference from zero using an alpha level of 0.05. Inflation of the type I error rate was avoided by treating each dose concentration group as a separate experiment.

RESULTS

Figure 3 is a box and whisker plot of the distribution of mean differences in DAT immunostaining, between matched pairs of permethrin-treated and vehicle control mice, within each of the "low dose" concentration groups. The tips of the whiskers respectively represent the minimum and maximum mean differences, while the length of the box represents the inter-quartile range. The line and solid square within each box respectively represent the median and grand mean of the distribution of mean differences. The number of matched pairs of treated and vehicle-control mice is also indicated for each dose. A grand mean of zero represents no change in immunostaining between matched pairs of mice. As can be seen in Figure 3, only the 3.0 mg/kg dose of permethrin produced a significant decrease in DAT immunostaining within the caudate-putamen, compared with matched vehicle control mice ($df = 7$, $p = .007$), although all "low dose" permethrin-treated mice showed a trend toward decreased labeling.

Figure 4 is a box and whisker plot of mean differences in DAT, TH an GFAP immunostaining, between matched pairs of treated and vehicle control mice, in the "high dose" concentration group (200 mg/kg permethrin). As seen in the figure, the "high dose" of permethrin did not produce a significant change in DAT or TH immunostaining within the caudate-putamen, compared with matched vehicle controls, although there was a trend toward a decrease for both types of labeling. However, as indicated by the asterisk, the "high dose" of permethrin resulted in a significant increase in striatal GFAP immunostaining, compared to matched vehicle controls ($df = 7$, $p = .048$).

DISCUSSION

The results of this experiment provide further support for the notion that environmental chemicals, in this case insecticides, can produce changes in components of the dopaminergic nigrostriatal pathway; a pathway that is the primary neurodegenerative substrate of Parkinson's disease. Taken together with a previous report on mouse striatal synaptosomes, these data indicate that a low dose (1.5-3.0 mg/kg) of the pyrethroid insecticide permethrin can not only alter the kinetics of dopamine uptake (Karen et al., 2001), but can change the amount of DAT immunoreactive protein within the caudate-putamen. Furthermore, high doses of permethrin, that also modify the kinetics of dopamine uptake, can induce glial responses in the caudate-putamen that have been associated with neuronal tissue damage. The lowest doses at which such changes are observed are more than two orders of magnitude lower than the LD_{50} reported for i.p. administration of the commercial formulation of permethrin known as Ambush (Williamson et al., 1989). Since there is evidence that technical grade permethrin is significantly less toxic than an equivalent amount delivered within the commercial mixture, it is likely that the lowest effective dose in this experiment was an even smaller proportion of the LD_{50} for technical grade permethrin (Williamson et al., 1989).

Although low doses of permethrin can affect both dopamine uptake and the amount of DAT immunoreactive protein in components of the dopaminergic nigrostriatal pathway, the effects are in different directions. A 1.5 mg/kg dose of permethrin has been reported to produce an increase in maximal dopamine uptake in striatal synaptosomes (Karen et al., 2001), while in the present experiment 3.0 mg/kg produced a decrease in DAT immunoreactive neuropil within the caudate-putamen. These two findings would be concordant if an increase in the efficiency of DAT transport overcompensated for a decrease in the amount of DAT protein. However, Karen et al. (2001) failed to find a change in the K_m for synaptosomal dopamine uptake, arguing against increased DAT efficiency.

Alternative explanations for the difference in the direction of the two findings noted above relate to differences in the nature of the tissue samples analyzed. For example, in the present immunohistochemical study, anatomical landmarks within transverse forebrain sections were used to select analysis fields within the caudate-putamen, the region of the striatum containing the vast majority of dopaminergic afferents from the substantia nigra (Heimer et al., 1995). Furthermore, each of these analysis fields was only on the order of 3070 μm^2. The kinetic data of Karen et al. (2001) was gathered in a synaptosomal preparation made from the entire dissected striatum. This sample may have included varying amounts of tissue from striatal regions outside the caudate-putamen, such as the nucleus accumbens or non-striatal regions such as the globus pallidus, both of which are physically contiguous with the caudate-putamen (Franklin & Paxinos, 1996). Therefore, the present study analyzed changes in a smaller sample of tissue, from a more topographically restricted region of the striatum; the region that is most closely associated with degenerative changes in PD (Kish et al., 1988; Graybiel et al., 1990; Miller et al., 1997). This suggests the possibility that different topographic sub-regions within the striatum may be differentially affected by permethrin exposure. Indeed, it has been shown that portions of the dopaminergic input to the ventral and medial striatum are more resistant to the neurodegenerative effects of MPTP and rotenone compared with the dopaminergic input to the more dorsolateral striatal region containing the caudate-putamen (Gerhardt et al., 1985; Mizukawa et al., 1990; Betarbet et al., 2000). In addition, using a whole striatal preparation of mouse synaptosomal membranes, it has recently been reported that low systemic doses of permethrin increased the amount of DAT protein as revealed by Western blot analysis (Bloomquist et al., 2002).

Although the sampling within the present experiment was more restricted with regard to striatal topography, the immunohistochemical analysis most likely surveyed a more extensive portion of individual nigrostriatal neuronal morphology compared with analysis of synaptosomes. Synaptosomal preparations are designed to isolate the synaptic bouton and sometimes contain a portion of the immediate post-synaptic membrane (Webster, 2001). Alternatively, any DAT-

containing portion of a neuron that lies near the surface of the striatal tissue section is available for labeling by the immunohistochemical procedure. Electron microscopic immunohistochemical studies of the dorsolateral striatum and substantia nigra pars compacta have reported that DAT immunoreactive protein can be found not only at the plasma membranes of synaptic boutons, but at the membranes of axonal segments lying between the boutons, as well as at somal and dendritic membranes of dopaminergic neurons (Nirenberg et al., 1996; Hersch et al., 1997). Since inter-bouton axonal segments course through the caudate-putamen, immunohistochemical procedures should label axon-associated DAT, in addition to bouton-associated DAT. Synaptosomal preparations would be expected to primarily sample bouton-associated DAT, since these structures do not contain axonal membrane, and any portion derived from the membrane of a post-synaptic striatal neuron would be almost exclusively non-dopaminergic. Therefore, the regional intra-neuronal population of DATs examined in the present study may have differed from that examined in studies using synaptosomal preparations from whole striatum (Karen et al., 2001; Bloomquist et al., 2002). In some dopaminergic neurons, there is a functional differentiation between DATs located in different regions of the cell. For example, dendritic DATs in the substantia nigra are capable of releasing dopamine, through reverse transport, under normal physiological conditions (Falkenburger et al., 2001). This potential for functional differentiation between DATs in different parts of the neuron, combined with a probable difference in intra-neuronal populations of DATs examined, could also account for the difference in direction of the kinetic data of Karen et al. (2001) and the present immunohistochemical study.

As noted in the Introduction, a decrease in maximal dopamine uptake (V_{max}) has been reported for striatal synaptosomes following higher doses of permethrin (25 or 200 mg) (Karen et al., 2001). This suggested that these doses may induce significant death of dopaminergic striatal terminals. The dopamine synthesizing enzyme TH is commonly used as an immunohistochemical marker to identify the presence of dopaminergic neuropil within the striatum (e.g. Gerhardt et al., 1985; Nirenberg et al., 1996, 1997; Ho and Blum, 1998; Brooks et al., 1999; Canudas et al., 2000; Betarbet et al., 2000). Although this enzyme is also critical for synthesis of norepinephrine and epinephrine, there is an insignificant presence of neurons that contain these transmitters within the striatum (Aston-Jones et al., 1995; Saper, 2000). Therefore, in the "high dose" portion of the present work, TH immunoreactivity was used as an additional marker for dopaminergic terminal death within the striatum since it was possible that the DAT protein could be down-regulated without accompanying degeneration of dopaminergic striatal afferents. The lack of change in the amount of DAT and TH immunoreactive neuropil, at doses of 200 mg/kg of permethrin, fails to support the notion that dopaminergic terminal death, within the caudate-putamen, is a substrate for the previously reported decrease in synaptosomal V_{max}. The absence of a decrease in DAT and TH immunoreactivities is consistent with a previous report that this dose did not change striatal levels of dopamine (Karen et al., 2001), which also argues against terminal death being responsible for the decrease in V_{max}.

Increased glial fibrillary acidic protein (GFAP), an intermediate filament protein of astrocytes, has been shown to be a marker of the onset, degree and locus of neuropathology (Norton, 1992; O'Callaghan, 1993; O'Callaghan et al., 1995; Eng et al., 2000). Such increases correspond not only to sites where there is easily identifiable loss of neuropil, such as the striatum following MPTP or MPP^+ exposure (Schneider and Denaro, 1988; Francis et al., 1995; Canudas et al., 2000; Akari et al., 2001), but where there is histological evidence of tissue damage that is more subtle than frank degeneration (O'Callaghan et al., 1995). Although the lack of change in TH and DAT immunoreactivities within the caudate-putamen argue against the death of dopaminergic nigrostriatal terminals in the "high dose" condition, the corresponding increase in GFAP immunoreactivity suggests the possibility of axon terminal damage that has not advanced to frank degeneration. Such damage may be sufficient to render the DAT inoperative, which would be consistent with the decrease in maximal dopamine uptake reported by Karen et al. (2001). Furthermore, non-fatal damage to the lipid bilayer of striatal synaptic boutons could permit non-transporter mediated leakage of dopamine from nigrostriatal terminals. This could also account for a decrease in

synaptosomal V_{max}, while leaving DAT and TH immunoreactivity unchanged. Again, such comparisons with the aforementioned synaptosomal study should be tempered by the possibility of a topographic difference in the striatal tissue samples examined. Furthermore, it should be noted that the observed increases in GFAP immunoreactivity could also represent damage to glial cells, since such increases have been reported following damage to oligodendrocytes and in astrocytes that survive direct astrocytic insult (Smith et al., 1983; Takada et al., 1990). Finally, there is evidence suggesting that MPTP-induced damage to the dopaminergic nigrostriatal pathway is capable of inducing an upregulation of tyrosine hydroxylase protein and mRNA (Greenwood et al., 1991; Bezard et al., 2000; Rothblat et al., 2001), as well as sprouting (Song and Haber, 2000), in surviving nigrostriatal neurons, across a variable time frame. If a high dose of permethrin were capable of a similar effect, a reduction in TH immunoreactivity, due to the death of nigrostriatal terminals, could be masked, while still inducing an increase in GFAP. This scenario cannot be ruled out. However, such compensatory effects within the nigrostriatal pathway may be dependent on the type of damage since lesions of this pathway, induced with 6-hydroxydopamine, produced a downregulation of TH mRNA in surviving nigral neurons (Sherman and Moody, 1995).

The present immunohistochemical data, combined with previous kinetic data, suggest that pyrethroid insecticides are capable of altering the normal functional status of the nigrostriatal pathway. The tissue damage suggested by increased GFAP within the caudate-putamen has obvious implications for the putative connection between pesticide exposure and Parkinson's disease. However, alterations of DAT protein within this region may also represent an important substrate modulating the onset and severity of Parkinson's disease since this transporter is the means by which Parkinson's disease-like inducing chemicals (e.g. MPP^+) can enter dopaminergic neurons (Mayer et al., 1986; Gainetdinov et al., 1997; Bezard et al., 1999). Such changes in the integrity of the DAT may be important for future investigations of the role of synergistic interactions between environmental chemicals in the development of Parkinson's disease.

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FIGURES

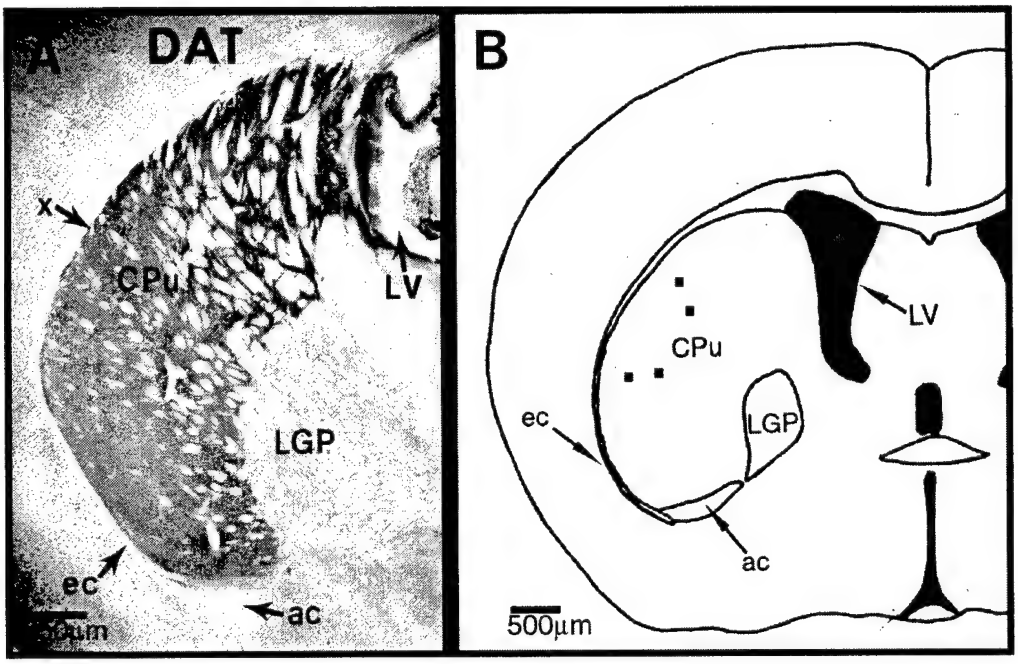

Figure 1. (A) Low magnification image of a coronal section through the striatum showing immunostaining for DAT. The region indicated by the "x" is shown at higher magnification in Fig. 2. (B) Schematic diagram of a similar section, modified from a mouse brain atlas (Franklin and Paxinos, 1996), showing the location and size (3070 μm^2) of each of the four analysis fields in the dorsal lateral portion of the striatum. Both images also illustrate prominent anatomical landmarks in the region. CPu=caudate-putamen, ec=external capsule, LV=lateral ventricle, ac=anterior commissure, LGP=lateral globus pallidus.

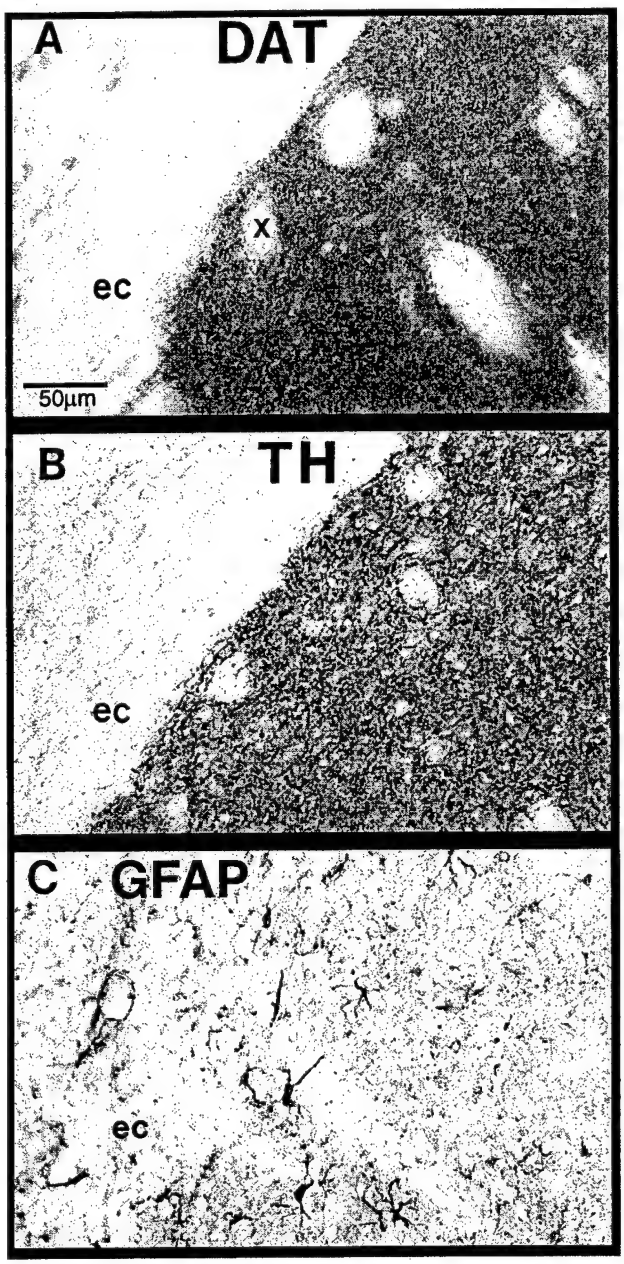

Figure 2. (A) Higher magnification image of a portion of the section shown in Fig. 1A. The " x " in both figures indicates the same tissue location. (B) and (C) Similar locations within the caudate-putamen, relative to 2A, showing staining for TH and GFAP, the other two antibodies used in the study. The anatomical landmark of the external capsule is indicated in all 3 photomicrographs. DAT=dopamine transporter, TH=tyrosine hydroxylase, GFAP=glial fibrillary acid protein, ec=external capsule.

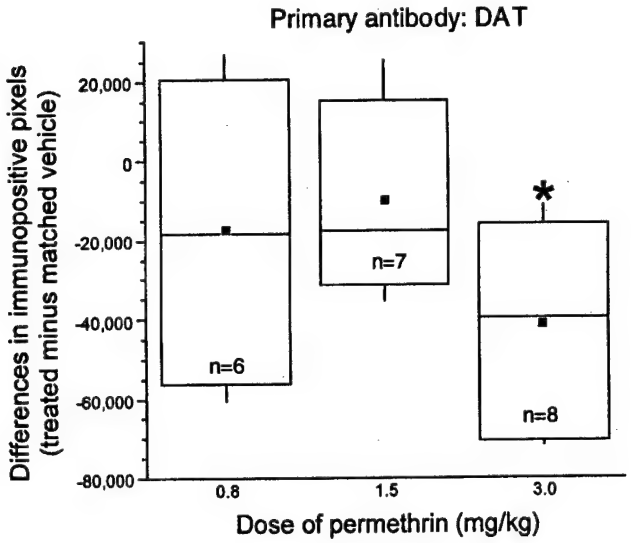

Figure 3. Distributions of differences in immunopositive staining for DAT, between pairs of pesticide-treated and matched vehicle control mice, for each of the low doses of permethrin. For each box and whisker plot, whisker tips respectively represent minimum and maximum differences while box length shows the inter-quartile range of the differences. The line and black square respectively indicate the median and mean of the difference scores. The asterisk indicates a mean of difference scores significantly different from zero. n=number of matched pairs of permethrin-treated and vehicle control mice.

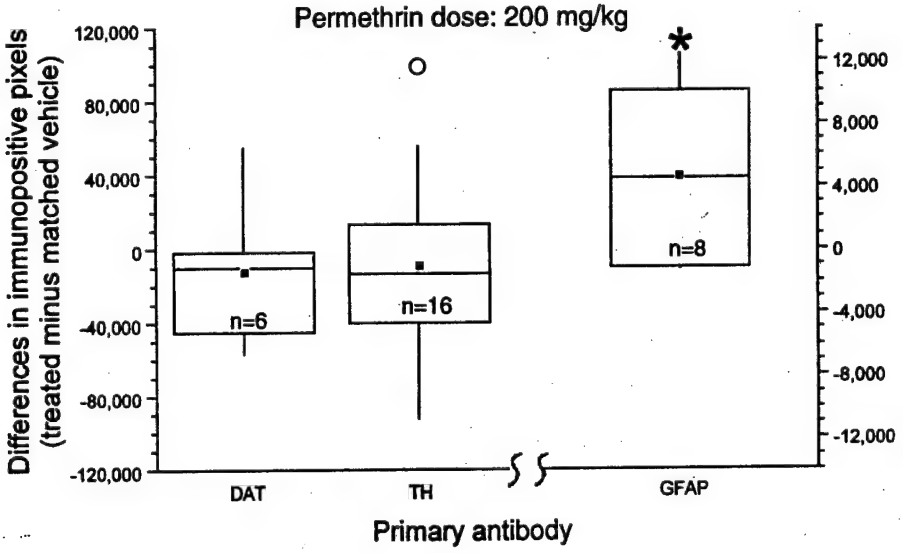

Figure 4. Box and whisker plots of differences in immunopositive staining for DAT, TH and GFAP, between matched pairs of pesticide-treated and vehicle control mice, for the high dose (200 mg/kg) of permethrin. The open circle is a difference score that is more than 1.5 inter-quartile ranges above the median. The asterisk indicates a mean of difference scores significantly different from zero. Note that the Y-axis for the GFAP data is drawn to a different scale since the amount of GFAP immunopositive material is normally an order of magnitude more sparse compared with that for DAT and TH.

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Striatal Dopaminergic Pathways as a Target for the Insecticides Permethrin and Chlorpyrifos

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Abstract

Because insecticide exposure has been linked to both Parkinsons disease and Gulf War illness, the neurotoxic actions of pyrethroid and organophosphate insecticides on behavior and striatal dopaminergic pathways were investigated in C57BL/6 mice treated with permethrin (three i.p. doses at 0.2–200 mg/kg) or chlorpyrifos (three s.c. doses at 25–100 mg/kg) over a 2-week period. Permethrin altered maximal [^{3}H]dopamine uptake in striatal synaptosomes from treated mice, with changes in V_{max} displaying a bell-shaped curve. Uptake was increased to 134% of control at a dose of 1.5 mg/kg. At higher doses of PM (25 mg/kg), dopamine uptake declined to a level significantly below that of control (50% of control at 200 mg/kg, $P < 0.01$). We also observed a small, but statistically significant decrease in [^{3}H]dopamine uptake by chlorpyrifos, when given at a dose of 100 mg/kg. There was no significant effect on the K_m for dopamine transport. Evidence of cell stress was observed in measures of mitochondrial function, which were reduced in mice given high-end doses of chlorpyrifos and permethrin. Although cytotoxicity was not reflected in decreased levels of striatal dopamine in either 200 mg/kg PM or 100 mg/kg CPF treatment groups, an increase in dopamine turnover at 100 mg/kg CPF was indicated by a significant increase in titers of the dopamine metabolite, 3,4-dihydroxyphenylacetic acid. Both permethrin and chlorpyrifos caused a decrease in open field behavior at the highest doses tested. Although frank Parkinsonism was not observed, these findings confirm that dopaminergic neurotransmission is affected by exposure to pyrethroid and organophosphorus insecticides, and may contribute to the overall spectrum of neurotoxicity caused by these compounds.

Keywords: Parkinson's disease; Dopamine transport; Pyrethroid; Organophosphate; Gulf War illness

INTRODUCTION

The pyrethroid and organophosphorus (OP) insecticides are members of two chemical classes of heavily used compounds, and hazards from exposure to insecticides exist from the manufacture, storage, spraying, and contact of personnel with insecticide-contaminated food or areas. Among the possible hazards is a consistent epidemiological linkage between insecticide exposure and the incidence of Parkinson's disease (Semchuk et al., 1992; Butterfield et al., 1993; Gorell et al., 1998). Further, the neurological health problems which comprise Gulf War illness, reported by over 30,000 veterans, may be due to exposure of personnel to various chemicals. Specific compounds implicated include pyridostigmine bromide, the insecticides permethrin (PM) and chlorpyrifos (CPF), and the repellent *N,N*-diethyl-*m*-toluamide (DEET) (Abou-Donia et al., 1996; Abou-Donia et al., 2001). In previous studies, we have documented effects of the pyrethroid deltamethrin (Kirby et al., 1999) and the organochlorine heptachlor (Bloomquist et al., 1999; Kirby et al., 2001) on dopaminergic nerve pathways, which might be a contributory factor in the etiology of environmentally-induced Parkinson's disease (PD). The present study assessed effects on dopamine pathways following exposure to PM and CPF to ascertain whether damage to dopaminergic pathways and attendant Parkinsonism

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might be a consequence of Gulf War chemical exposures.

MATERIALS AND METHODS

Chemicals

Analytical grade CPF was obtained from ChemService Inc. (West Chester, PA). PM (a mixture of four *R,S-cis* and *R,S-trans* isomers) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were obtained from Sigma Chemical Co., St. Louis, MO, USA. [^{3}H]Dopamine (20.3 Ci/mmol) was purchased from NEN Life Science Products Inc., Boston, MA, USA. Choline-Cl, KCl, $MgCl_2$, $CaCl_2$, and ascorbate were obtained from Sigma Chemical Co., Pargyline, D-glucose, sucrose, and HEPES were obtained from Fisher Scientific Co., Pittsburgh, PA, USA.

Animals and Treatments

Male C57BL/6 retired breeder mice were utilized for all experiments. Mice were purchased from Harlan Sprague–Dawley, Dublin, VA, USA and were aged 7–9 months (28–40 g live weight) at the time of the experiments. Mice were assigned randomly to treatment groups, which contained a minimum of six mice, so that the mean weight of all treatment groups was approximately equal. CPF carried in corn oil vehicle or PM carried in methoxytriglycol (MTG) vehicle were administered to the mice at multiple doses three times over a 2-week period according to the method of Bloomquist et al. (1999). CPF administration was by s.c. injection, while PM was administered by i.p. injection. Control mice received 50 μl corn oil or 10 μl of MTG vehicle alone. On the day following the last treatment day, mice were killed by cervical dislocation, and striatal tissues were collected at this time.

Dopamine Uptake Studies

Labeled dopamine uptake studies were performed according to the method outlined in Kirby et al. (1999). Briefly, crude synaptosomes were prepared from fresh striatal tissue dissected from treated mice, and incubated with [^{3}H]dopamine at various concentrations for 2 min. Transport of dopamine was determined after washing and vacuum filtration, followed by liquid scintillation counting. Uptake rates were determined by the method of Krueger (1990) in incubations with and without sodium ions (equimolar choline chloride substitution) in order to correct for low affinity transport. Uptake parameters (V_{max} and K_m) were determined by nonlinear regression to isotherm plots (Prism™, GraphPad Software, San Diego, CA, USA). Aliquots of each synaptosomal preparation were frozen at −20°C for membrane protein determinations, which was according to the method of Bradford (1976).

MTT Cytotoxicity Assay

This assay was run on synaptosomes by adapting the cultured cell methods of Carmichael et al. (1987) to synaptosomes. Striatal synaptosomes were prepared as described in Kirby et al. (1999) and incubated with MTT dissolved in Krebs–Henseleit buffer containing (mM): NaCl (140), KCl (5.0), $MgSO_4$ (1.3), $NaHCO_3$ (5.0), Na_2HPO_4 (1.0), HEPES (10), glucose (10), and $CaCl_2$ (1.2), pH 7.4. After 30 min at 37°C, the tubes were centrifuged for 5 min at 10,000 × *g*. The pellets were resuspended in DMSO to solubilize the formazan reduction product, and centrifuged again at 10,000 × *g* for 1 min. Background absorbance of MTT (650 nm) was subtracted from test absorbance (580 nm) for the blue formazan product, both determined by a 96-well plate reader (Dynex Technologies Inc., Chantilly, VA, USA).

Dopamine and DOPAC Content

The methods employed were similar to those of Hall et al. (1992). Striata from individual mice were homogenized in 5% TCA containing 10 ng dihydroxybenzylamine (DHBA)/mg tissue wet weight as an internal standard and frozen at −70°C until analysis. Prior to analysis, samples were thawed and centrifuged at 10,000 × *g* to pellet tissues. Dopamine and its metabolite 3,4-dihydroxyphenylacetic acid (DOPAC) were separated by HPLC using an ODS 3 μm Phase 2 column (3.2 mm × 100 mm). Mobile phase consisted of 170 mM NaH_2PO_4, 1.5 mM octanesulfonic acid, 5.5% methanol, 1.5% acetonitrile, and 93% H_2O, at a flow rate of 0.6 ml/min. Dopamine, DOPAC and DHBA standards were prepared to quantitate the amounts of dopamine and DOPAC in the samples.

Behavioral Assessments

On the last day of the study and prior to neurochemical analyses, behavioral effects were assessed by means of monitoring the number of open field movements and rearing frequency in an arena over a 3-min

period. The open field was the floor of a 10 gallon aquarium, divided into six equally sized squares. A movement was counted when the animal's front paws crossed any grid line. A rear was counted when the mouse raised onto its rear paws, lifting the front paws from the floor of the field.

Statistical Analysis

Statistical significance was determined using one-way ANOVA and Student–Newman–Keuls means separation if a statistically significant main effect of treatment was observed. Other statistical comparisons were by *t*-test calculations performed using InStat™ (GraphPad Software).

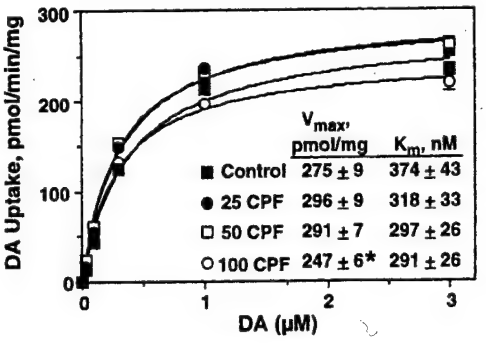

Fig. 2. Effect of CPF treatment on dopamine uptake in ex vivo striatal synaptosomes. Symbols represent means of three determinations with bars equal to the S.E.M. Absence of bars means that the S.E.M. was less than the size of the symbol. Kinetic and statistical analysis is given in the inset table. Asterisks indicate an effect significantly different from control (*t*-test, $P < 0.05$).

RESULTS

Dopamine uptake rate in striatal synaptosomes displayed the expected saturation with increasing concentration of substrate (Fig. 1A). All curves showed a good fit to a Michaelis–Menten model, and typically had correlation coefficient (r^2) values $\geq$0.98. PM treatment did not have any significant effect on the apparent K_m of the dopamine transporter in any of the treatment groups. The control values for K_m averaged 233 ± 28 nm (mean ± S.E.M.). Maximal dopamine uptake in 9-month-old C57BL/6 mice treated with PM did vary with the dose, increasing at lower doses of PM, while at higher doses maximal uptake declined until it was less than the control level (Fig. 1A). Thus, a bar graph (Fig. 1B) of PM-induced V_{max} values from a broader range of doses took the form of a bell-shaped curve, in which the maximal rate of dopamine uptake, V_{max}, peaked at a dose of 1.5 mg/kg. At this dose, dopamine uptake was significantly greater (34%) than that of the control value, and this dose was replicated in four different groups of mice. All other doses were replicated at least twice. At higher doses of PM (>25 mg/kg), V_{max} declined to a level significantly below that of control (50% of control at 200 mg/kg) (Fig. 1B). Treatment of mice with CPF also caused a reduction in dopamine uptake V_{max} at the highest dose administered (100 mg/kg) (Fig. 2). Doses of CPF below 25 mg/kg were not tested in these experiments.

Striatal MTT dehydrogenase activity, an assay of mitochondrial integrity, was performed on pooled membranes from treated mice. Production of reduced formazan was reduced by 100 mg/kg, but not 50 mg/kg of CPF, which was actually higher than control (Fig. 3). The reduction caused by 100 mg/kg CPF was present at all concentrations of thiazolyl blue (MTT) tested, although only data at 0.55 mM is shown. PM also reduced MTT dehydrogenase activity, but at lower doses than CPF. At doses of 12.5, 25 and 50 mg/kg

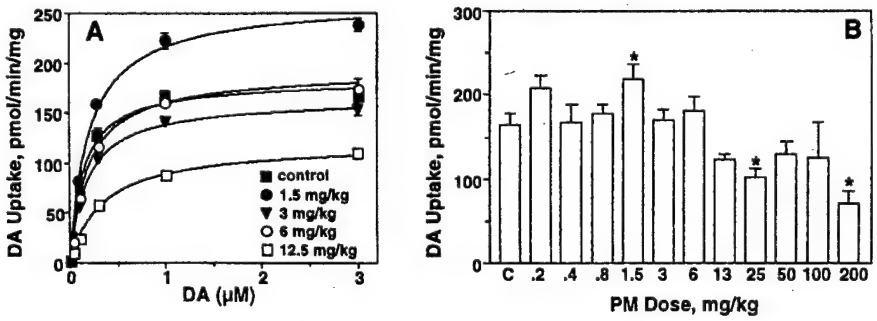

Fig. 1. Representative isotherm plots (A), and a bar graph (B) of the effects of PM on dopamine uptake. (A) Symbols represent means of three determinations with bars equal to the S.E.M. Absence of bars means that the S.E.M. was less than the size of the symbol. (B) Percentage changes in maximal dopamine uptake (V_{max}) following PM treatment at the indicated doses. Asterisks indicate effects significantly different from control (*t*-test, $P < 0.05$).

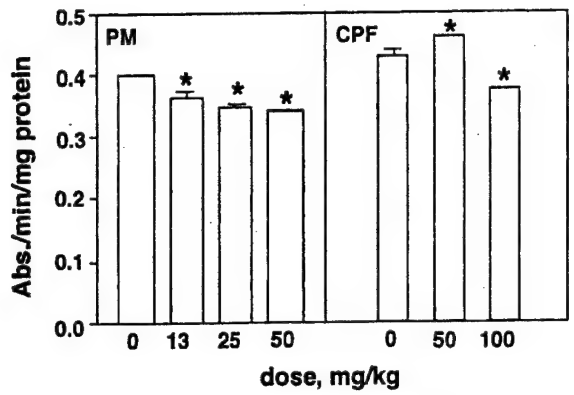

Fig. 3. Effect of PM (left) and CPF (right) treatment on mitochondrial activity (MTT reduction) in ex vivo striatal synaptosomes. MTT reduction activity is shown at a single concentration of 0.55 mM. In the bar graphs, the asterisk indicates an effect significantly different from control (ANOVA, $P < 0.05$).

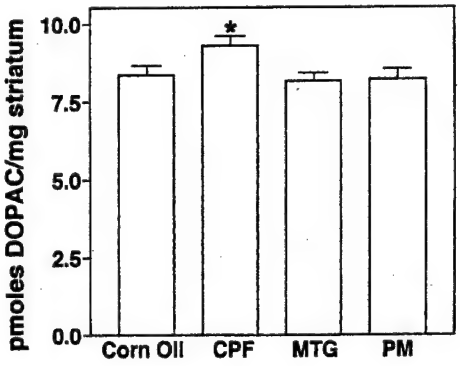

Fig. 4. Changes in DOPAC titers following treatment with vehicle (corn oil; MTG) or insecticide (100 mg/kg CPF; 200 mg/kg PM). Asterisks indicate effects significantly different from control (*t*-test, $P < 0.05$).

PM, MTT dehydrogenase activity was depressed 9, 12, and 14%, respectively. Similarly, there was a statistically significant 9% decrease in mitochondrial activity in a separate group of mice given 200 mg/kg PM.

CPF (100 mg/kg) increased striatal dopamine turnover, as indicated by significantly elevated titers of the dopamine metabolite, DOPAC (Fig. 4). The effect of CPF at this dose was an increase of 14% above control. In contrast, a high dose of PM (200 mg/kg) did not increase DOPAC titers. Neither CPF nor PM at these doses had any effect on striatal dopamine levels (data not shown).

CPF and PM both had similar dose-dependent effects on mouse behavior, according to movement and rearing tests. Statistically significant effects on rearing and movement were observed only at the highest doses of both compounds. Both movement and rearing frequency were decreased by treatment with 50 and 100 mg/kg CPF; however, the decrease at 50 mg/kg was not statistically significant (Fig. 5A and B). Similarly, high doses of PM decreased both frequency of open field movement and rearing frequency (Fig. 6A and B). The effect was only significant at the 50 and 200 mg/kg doses, and not the 100 mg/kg dose, however.

DISCUSSION

The effect of 1.5 mg/kg PM for increasing the maximal transport of dopamine uptake is a potent action of this compound, in vivo. This dose is about three orders of magnitude below the rat oral LD_{50} for PM (Budavari et al., 1996), and we never observed any lethality at the highest dose (200 mg/kg) used in this study. Moreover, technical permethrin is a mixture of four (1*R*,*S-cis* and 1*R*,*S-trans*) isomers, only one of which (1*R*,-*cis*) has lethal effects in mammals (Casida et al., 1983). If the 1*R*,-*cis* isomer is responsible for the up-regulation, it is only 25% of the applied dose, and was actually given at about 0.4 mg/kg. We assume that the observed increase in dopamine uptake was compensatory for permethrin-dependent increases in dopamine release, in vivo. We have recently shown that the related pyrethroid deltamethrin releases a variety of neurotransmitters from preloaded synaptosomes, with the EC_{50} for dopamine release (48 nM) being 2.4- and

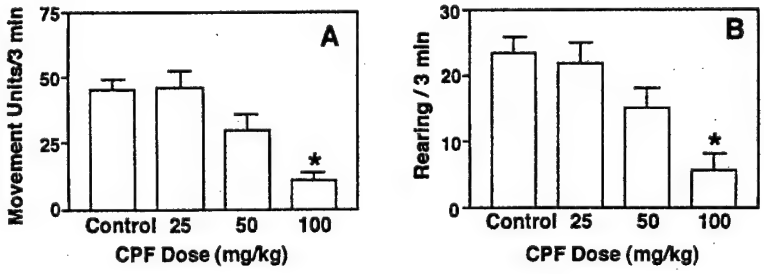

Fig. 5. Changes in open field (A), and rearing frequency (B) at the indicated doses of CPF. Asterisk indicates an effect significantly different from control (*t*-test, $P < 0.05$).

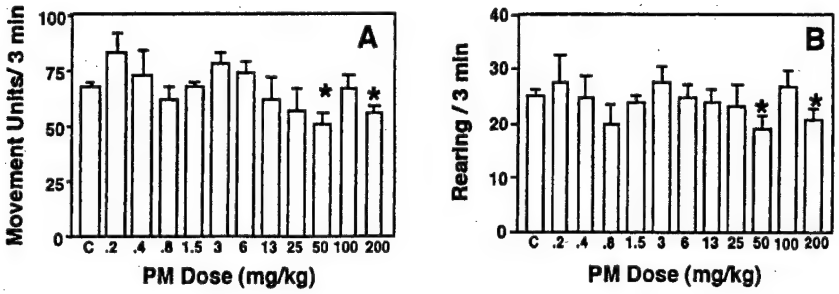

Fig. 6. Changes in open field (A), and rearing frequency (B) at the indicated doses of permethrin. Asterisk indicates an effect significantly different from control (*t*-test, $P < 0.05$).

8.6-fold more potent than serotonin or glutamate release, respectively (Kirby et al., 1999). Thus, increased dopamine outflow was presumably balanced by increased uptake to maintain normal dopaminergic neurotransmission.

As the dose of PM increased, maximal transport of dopamine decreased to a level about 50% below that of controls, most likely from an inability of the synaptosomes to retain dopamine, rather than a true effect on dopamine transport. We would expect degeneration of the nerve terminals to be reflected in loss of striatal dopamine, which was not observed. However, there was evidence of cell stress in mice treated with doses $\geq$12.5 mg/kg PM in the MTT assay, which is a measure of mitochondrial function (Carmichael et al., 1987). In future studies, we expect to observe an up-regulation of dopamine transporter (DAT) protein in western blots at doses near 1.5 mg/kg of PM. Our previous work has shown that the organochlorine insecticide heptachlor increases dopamine transport in male C57BL/6 mice about two-fold at a dose of 6 mg/kg and this increase in uptake was accompanied by an increase in DAT protein labeling in western blots of striatal membranes (Miller et al., 1999). Moreover, the dose-response curve for heptachlor has a shape similar to that reported here for PM (Kirby et al., 2001). We have also demonstrated this effect for the pyrethroid insecticide deltamethrin, which increased dopamine uptake by 70% following three doses of 6 mg/kg (Kirby et al., 1999).

In contrast to PM, striatal dopamine uptake is not up-regulated by lower doses of CPF; however, in these experiments doses under 25 mg/kg CPF were not tested. At higher doses of CPF (100 mg/kg), dopamine transport V_{max} is significantly decreased, as was the case for high doses of PM. Similarly, at a dose of 100 mg/kg CPF, MTT dehydrogenase activity is significantly depressed compared to controls, suggesting chemically-induced nerve terminal stress.

CPF and PM failed to have an effect on striatal dopamine titers at the relatively high doses administered (data not shown). However, incipient effects on dopamine could be occurring that are masked when measured as total amount of striatal dopamine by HPLC. The effect may be similar to that seen in aged mice, in which 68% of the dopaminergic neurons are lost naturally, but there is a 103% increase in dopamine synthesis by the remaining neurons as a compensatory effect (Tatton et al., 1991). DOPAC levels were increased by treatment with a high dose of CPF, but not PM. Loss of dopamine and DOPAC is a cardinal sign of PD (Hornykiewicz and Kish, 1987) and can reflect changes in both cellular levels of dopamine and cell death in the striatum. Elevated levels of DOPAC indicate greater turnover of dopamine in response to toxicant-induced processes (Hudson et al., 1985). We assumed that CPF increased turnover through neuronal hyperexcitation caused by inhibition of acetylcholinesterase, although interaction with other targets cannot be ruled out. The related compound methyl parathion, given at low doses (0.1 mg/kg per day for 15 days) to neonatal rats had little or no effect on dopamine content (Kumar and Desiraju, 1992). Soman induced an increase in DOPAC levels, consistent with an increase in dopamine turnover, but no change in dopamine levels (el-Etri et al., 1992; Fosbraey et al., 1990). We were somewhat surprised by the lack of any effect of PM on DOPAC, given that increased levels of striatal DOPAC had been demonstrated with this compound previously (Doherty et al., 1988). However the dose Doherty et al. used (1200 mg/kg, p.o.) probably gave a greater effective brain concentration than the treatment we used in this study (200 mg/kg, i.p.).

Changes in movement and rearing behaviors observed after CPF treatment are most likely due to inhibition of acetylcholinesterase activity, which is a hallmark of organophosphate exposure (Bowman and

Rand, 1980). At doses above 25 mg/kg, there is a good correlation between dose and impairment of movement and rearing. PM has a less clear dose-dependent effect on behavior than CPF. However, at doses above 50 mg/kg, PM decreases both movement and rearing frequencies. This action is consistent with results reported by Spinosa et al. (1999), in which movement and rearing frequencies were reduced by 10 and 30 mg/kg doses of the pyrethroid fenvalerate, which has greater mammalian toxicity than PM (Budavari et al., 1996). Although bradykinesia is a hallmark of PD (Bowman and Rand, 1980), it was not accompanied by a reduction in striatal dopamine in PM-treated mice, so another mechanism is probably responsible.

We have shown that up-regulated dopamine transport and mitochondrial integrity assays are sensitive biomarkers of exposure to certain insecticides. However, we do not know whether the neurochemical effects observed are persistent, or only temporary changes occurring after the last insecticide treatment. The lack of any effect on dopamine titers does not support a rapidly developing Parkinsonism following short-term exposures to these compounds. Long-term exposure studies should be undertaken. A recent study (Lee et al., 2001) observed an interaction between the DAT and α-synuclein in cultured neurons, which led to increased dopamine uptake and dopamine-induced cellular apoptosis. Such a scenario would provide a mechanism whereby increased DAT expression by insecticides could play role in the development of Parkinsonism.

Future studies will determine the extent of synergism in the effects of these insecticides on the nigrostriatal dopaminergic pathway, since synergistic neurotoxicity has been observed with DEET, pyridostigmine bromide, chlorpyrifos, and permethrin in various combinations (Abou-Donia et al., 1996; Abou-Donia et al., 2001). Other studies will address the extent of reversibility of the observed PM and CPF effects on striatal neurochemistry.

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Selective Effects of Insecticides on Nigrostriatal Dopaminergic Nerve Pathways

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Abstract

A degeneration of the nigrostriatal pathway is a primary component of Parkinson's disease (PD), and we have investigated the actions of insecticides on this pathway. For in vivo exposures, C57BL/6 mice were treated three times over a 2-week period with heptachlor, the pyrethroids deltamethrin and permethrin, or chlorpyrifos. One day after the last treatment, we observed that heptachlor and the pyrethroids increased maximal [^{3}H]dopamine uptake in striatal synaptosomes from treated mice, with dose-dependent changes in V_{max} displaying a bell-shaped curve. Western blot analysis confirmed increased levels of dopamine transporter (DAT) protein in the striatum of mice treated with heptachlor and permethrin. In contrast, we observed a small, but statistically significant decrease in dopamine uptake by 100 mg/kg chlorpyrifos. For heptachlor, doses that upregulated DAT expression had little or no effect on serotonin transport. Permethrin did cause an upregulation of serotonin transport, but required a 30-fold greater dose than that effective on dopamine uptake. Other evidence of specificity was found in transmitter release assays, where heptachlor and deltamethrin released dopamine from striatal terminals with greater potency than other transmitter types. These findings confirm that insecticides possess specificity for effects on striatal dopaminergic neurotransmission.

Keywords: Parkinson's disease; Permethrin; Deltamethrin; Heptachlor; Chlorpyrifos; Dopamine transporter; Dopamine release

INTRODUCTION

Parkinson's disease (PD) results mainly from a degeneration of dopaminergic fibers of the nigrostriatal pathway (Bowman and Rand, 1980). Among the possible causes of PD, there is a consistent epidemiological linkage with pesticide exposure (Semchuk et al., 1992; Butterfield et al., 1993; Gorell et al., 1998). Hazards from exposure to heavily used insecticides, such as the pyrethroids and organophosphates, exist from the manufacture, storage, spraying, and contact of personnel with insecticide-contaminated food or areas. Similarly, human exposure to organochlorine insecticides continues to be an important issue in human health, since the environmental persistence of these compounds has raised concerns regarding their documented ability for bioaccumulation (Matsumura, 1985).

Several studies have observed effects of organochlorines consistent with Parkinsonism. Early studies showed that rats fed 50 ppm dieldrin for 10 weeks had small reductions in whole brain dopamine after 4 weeks, but no change in the amount of striatal dopamine, even though striatal serotonin and norepinephrine levels were depressed (Wagner and Greene, 1974). Similarly, Mallard ducks (Sharma, 1973) and ring

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doves (Heinz et al., 1980) fed dieldrin also displayed significant reductions in brain dopamine. Chemical analysis of Parkinsonian human brain found that it contained significantly greater levels of organochlorines, especially dieldrin, than healthy brain (Fleming et al., 1994; Corrigan et al., 1998). In studies with mesencephalic neuron cultures, dieldrin application caused cytotoxicity in dopaminergic neurons more so than GABAergic neurons, suggesting some selectivity of action (Sanchez-Ramos et al., 1998).

We observed that the organochlorine insecticide heptachlor upregulated dopamine transport in striatal synaptosomes from treated mice at relatively low doses (Kirby and Bloomquist, 1996). Subsequent work expanded these findings, and showed that organochlorine and pyrethroid insecticides affect dopamine transporter (DAT) expression and dopamine release in the striatum (Kirby et al., 1999, 2001; Karen et al., 2001). The present paper will review our published work on these insecticides, and provide additional data confirming and extending our observations of the selective actions of insecticides on dopaminergic pathways.

MATERIALS AND METHODS

Chemicals

Analytical grade chlorpyrifos (≥99%) was obtained from Chem Service Inc. (West Chester, PA). Permethrin (a mixture of four *R, S-cis* and *R, S-trans* isomers) was obtained from Sigma (St. Louis, MO), and deltamethrin (a single, 1*R-cis*, α-*S* isomer) was supplied by Crescent Chemical Co. (Hauppauge, NY). Analytical grade (≥99% purity) heptachlor was purchased from Chem Service (West Chester, PA). [^{3}H]Dopamine (20.3 Ci/mmol) and [^{3}H]GABA (100 Ci/mmol) were purchased from New England Nuclear (Wilmington, DE). [^{3}H]Glutamate (56.0 Ci/mmol) and [^{3}H]serotonin (17.9 Ci/mmol) were purchased from Amersham (Buckinghamshire, UK). Buffer constituents were obtained from Sigma or Fisher Scientific Co. (Pittsburgh, PA).

Animals and Treatments

For experiments on insecticide-dependent changes in transporter regulation, we used male C57BL/6 retired breeder mice, purchased from Harlan Sprague-Dawley (Dublin, VA) that were 7–9 months old (28–40 g live weight). Treatments were administered three times over a 2-week period according to the method of Kirby et al. (1999). All insecticides were delivered in methoxytriglycol (MTG) vehicle and injected i.p., except CPF, which was carried in corn oil vehicle and given s.c. Control mice received 50 μl corn oil or 10 μl of MTG vehicle alone. On the day following the last treatment, mice were killed by cervical dislocation and tissues were collected for neurochemical analysis.

Dopamine and Serotonin Uptake Studies

Neurotransmitter uptake studies were performed according to the methods of Kirby et al. (1999) for dopamine and Kirby et al. (2001) for serotonin. Briefly, crude synaptosomes were prepared from fresh striatal (for dopamine) or cortical tissue (for serotonin) dissected from treated mice. Synaptosomes were incubated with various concentrations of [^{3}H]dopamine or [^{3}H]serotonin for 2 min. Uptake was stopped by addition of ice cold buffer, followed by vacuum filtration, washing, and liquid scintillation counting. Aliquots of each synaptosomal preparation were frozen at −20 °C for membrane protein determination, which was performed according to the method of Bradford (1976). Uptake rates were calculated by the method of Krueger (1990), using incubations with and without sodium ions (equimolar choline chloride substitution) to correct for nonspecific uptake. Uptake parameters (V_{max} and K_m) were determined by nonlinear regression to isotherm plots using Prism™ (GraphPad Software, San Diego, CA).

Western Blot Analysis

Western blots were used to quantify the amount of DAT protein present in samples of brain striatal tissue from treated mice. Crude synaptosomal membranes were prepared as previously described (Kirby et al., 2001) and then homogenized, heat denatured, and the proteins separated by SDS–PAGE electrophoresis (Laemmli, 1970). Separation was accomplished on a 10% SDS–PAGE gel (10 μg protein per lane) and transferred electrophoretically to a nitrocellulose membrane for 1 h at 100 V, according to the method of Towbin et al., 1979). The nitrocellulose membranes are stained with Ponceau S dye to confirm transfer of the proteins to the membrane, and it serves the added purpose of ensuring that equal amounts of protein have been loaded onto each lane. Blots were then incubated in 4% dry milk for 1 h, followed by a 1 h incubation in anti-DAT antibody (rat, Chemicon, Temecula, CA) diluted 1:1000, and 1 h with peroxidase-linked secondary antibodies. The blots were visualized using the ECL

Chemiluminescense detection kit (Amersham, Buckinghamshire, England), with exposure to the ECL reagent for 1 min, followed by autoradiography for various lengths of time, up to 2 min. Analysis of the blots was performed by digital image analysis using a Kodak Digital Camera and EDAS 290 System (Eastman Kodak Scientific Imaging Systems, Rochester, NY).

Neurotransmitter Release

Conventional neurotransmitter release assays in striatal or cortical synaptosomes were performed essentially as described in Kirby et al. (1999). Crude synaptosomes were prepared from the two tissue sources and the final pellets resuspended in incubation buffer containing either 100 nM [^{3}H]dopamine, 115 nM [^{3}H]serotonin, 40 nM [^{3}H]GABA or 90 nM [^{3}H]glutamate (5 min, 37 °C). Cortical tissue was used as a synaptosome source for assays with [^{3}H]serotonin, due to the relatively low density of serotonergic terminals in the striatum. After loading, the membranes were centrifuged and the labeled pellets resuspended in buffer and incubated with toxicants for 10 min at 37 °C. Lipophilic toxicants were dissolved in DMSO and final DMSO concentrations in incubations did not exceed 0.1%, with controls receiving 0.1% DMSO alone. Synaptosomes were diluted with 3 ml of wash buffer (37 °C), vacuum-filtered, and then washed three times with 3 ml of 37 °C wash buffer. Radioactivity on the filters was determined as described before.

Statistical Analysis

For uptake studies, kinetic parameters (K_m and V_{max}) were determined by nonlinear regression to isotherm plots (GraphPad Software, San Diego, CA). For release studies, data were analyzed by nonlinear regression to a four-parameter logistic equation using either Prism™ 2.0 (GraphPad Software, San Diego, CA) or MacCurveFit 1.3 (Kevin Raner Software, Vic., Australia), which gave similar results. EC_{50} values for release of different neurotransmitters or dopamine uptake parameters following insecticide treatment were compared by *T*-test or by one-way ANOVA with Student–Newman–Keuls means separation test (InStat 2.03, GraphPad Software, San Diego, CA).

RESULTS

Dopamine uptake in striatal synaptosomes from treated mice was saturable and showed a good fit to a Michaelis–Menten model (r^2 values ≥ 0.98). Permethrin treatment did not have any significant effect on the apparent K_m of the DAT (range: 375–545 nM) in any of the treatment groups. Maximal dopamine uptake in C57BL/6 mice treated with permethrin varied with the dose (Fig. 1A), and peaked at a dose of 1.5 mg/kg. At this dose, dopamine uptake was significantly greater (34%) than that of the control value, while at higher doses maximal uptake declined. A similar dose-response profile was observed for heptachlor. Increases in V_{max} had a threshold dose of 3 mg/kg and uptake was maximally enhanced at 6 mg/kg (Fig. 1B). Maximal induction of V_{max} for striatal dopamine transport was 217% of control at this dose. Again, at higher doses, transport declined. Changes in apparent K_m did not match in a dose-dependent way the increases in V_{max} and displayed a variable response to heptachlor treatment (Kirby et al., 2001). In contrast, treatment of mice with chlorpyrifos caused a significant 10% reduction in dopamine uptake V_{max} at 100 mg/kg, the highest dose administered. No significant effect on V_{max} was observed at lower doses of chlorpyrifos.

Upregulation of the DAT was confirmed in striatal tissue taken from treated mice. We observed that two major protein bands were labeled by DAT antibody (Fig. 2, top). Scanning densitometry found clear evidence of greater antibody labeling, especially at the 0.8 mg/kg dose of permethrin. In Fig. 2 (bottom), both bands were upregulated, with the total effect of about a 30% increase in labeling of each band. In addition, we have observed that permethrin-dependent upregulation

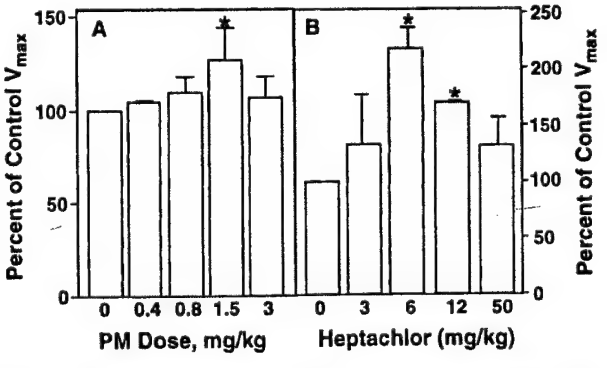

Fig. 1. Representative bar graphs of dopamine transport after permethrin (A) or heptachlor treatment (B). Bars represent mean (with S.E.M.) changes in V_{max} values, expressed as percent of control and replicated across different cohorts of treated mice. Asterisks indicate effects significantly different from control using the untransformed data (*T*-test, $P < 0.05$). The permethrin plot (A) is redrawn from Karen et al. (2001) and the heptachlor plot (B) is redrawn from Kirby et al. (2001).

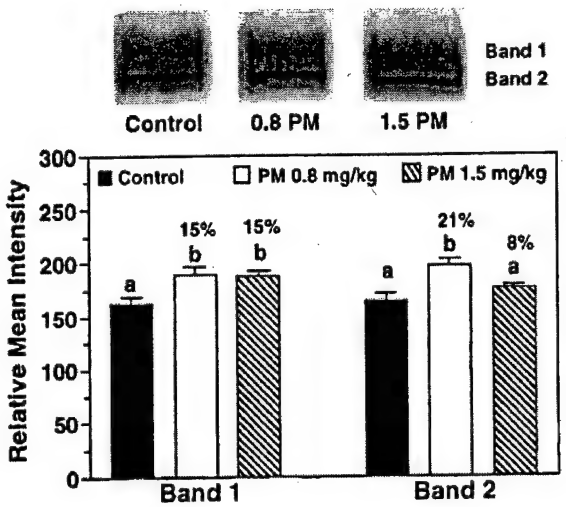

Fig. 2. Western blot of striatal membranes taken from mice treated with the indicated doses of permethrin (PM) three times over a 2-week period. Top: each lane shows two major protein bands, and is from a pooled striatal homogenate isolated from treated mice. Bottom: scanning densitometry of the bands shows an increase in DAT protein, with the percentage increase from matched controls given above the bars for the PM treated groups. Bars labeled by different letters are significantly different from controls (*T*-test, $P < 0.05$).

of the DAT is still present 4 weeks after the last 0.8 mg/kg treatment (data not shown).

Levels of cortical serotonin uptake following heptachlor treatment did not reflect the increase in maximal uptake observed for dopamine (Fig. 3). These experiments used 6 and 12 mg/kg heptachlor; the doses that in previous experiments produced the greatest increases in V_{max} for dopamine uptake. In these studies, there was no significant change in V_{max} from control (5.1 ± 0.1 pmol/min/mg) in mice treated with 6 mg/kg heptachlor (5.0 ± 0.2 pmol/min/mg). There was a statistically significant, 21% reduction in calculated maximal rates of serotonin uptake for 12 mg/kg heptachlor-treated mice (4.1 ± 0.3 pmol/min/mg). However, this reduction was essentially attributable to a more variable decrease in uptake at 1 μM serotonin only, since the other points on the curve (Fig. 3) overlapped closely those of the other treatment groups. No statistically significant change in K_m values was measured for serotonin uptake (range: 90–140 nM) in any of the heptachlor-treated mice.

Reduced sensitivity of cortical serotonergic pathways was also observed with permethrin treatment. Ex vivo cortical synaptosomes showed a dose-dependent upregulation of serotonin transport (Fig. 4), but at doses at least 30-fold greater than that required to upregulate dopamine uptake. Upregulation was essentially complete at 100–200 mg/kg permethrin, and virtually disappeared as the dose was lowered to 25 mg/kg (Fig. 4). In addition, the maximal extent of upregulation of serotonin transport (34%), was identical to that observed for dopamine. There was no statistically significant effect on the K_m values for serotonin transport (range: 69–289 nM).

Differential sensitivity of nerve terminals to pyrethroid- or heptachlor-evoked release of neurotransmitter was observed in synaptosome preparations from striatum and cortex of ICR mice (Table 1). The pyrethroid deltamethrin released a variety of neurotransmitters

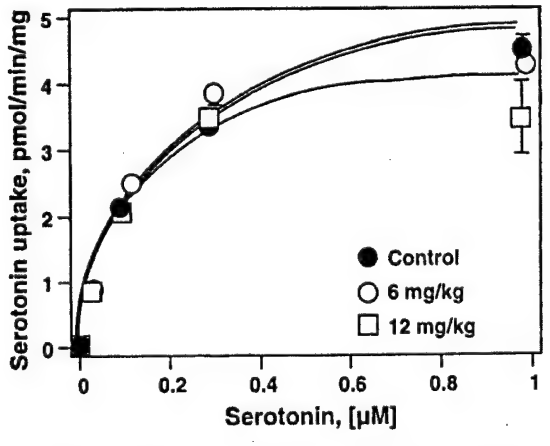

Fig. 3. Serotonin uptake following treatment at doses of heptachlor known to upregulate dopamine transport. Symbols indicate means, along with the S.E.M. Lack of error bars indicates that the S.E.M. was smaller than the size of the symbol. Redrawn from Kirby et al. (2001).

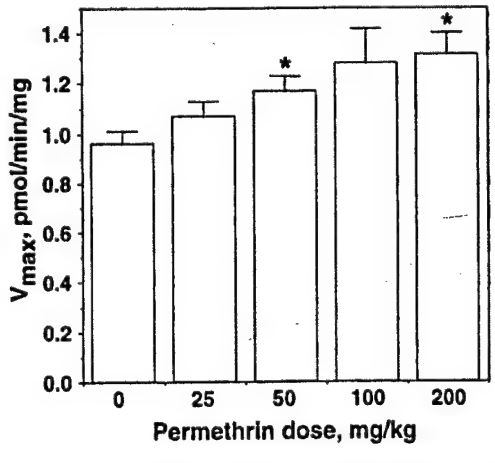

Fig. 4. Serotonin uptake in cortical synaptosomes following treatment with permethrin. The results are taken from pooled membranes of single cohorts of mice having typically five to six animals. Asterisks indicate serotonin transport significantly different from control (*T*-test, $P < 0.05$).

Table 1
Potency of insecticides for releasing different neurotransmitters from synaptosomes

Insecticide	Brain region	Transmitter	EC_{50} ± S.E.M.[a]
Deltamethrin[b]	Striatum	Dopamine	48[a] ± 25 nM
	Cortex	Serotonin	117[b] ± 37 nM
	Cortex	Glutamate	412[b] ± 76 nM
Heptachlor	Striatum	Dopamine	1.1[a] ± 1.1 μM
	Striatum	GABA	7.3[b] ± 1.2 μM
	Striatum	Glutamate	13.7[c] ± 1.2 μM
	Cortex	Serotonin	25.9[d] ± 1.3 μM

[a] EC_{50} values labeled by different letters are significantly different from each other, as found by one-way ANOVA and Student–Neumann–Keuls means separation test ($P < 0.05$).
[b] Data taken from Kirby et al. (1999).

from preloaded synaptosomes at nanomolar concentrations, with the EC_{50} for dopamine release being 2.4- and 8.6-fold more potent than serotonin or glutamate release, respectively. The EC_{50} for heptachlor-evoked release of [^{3}H]dopamine from striatal synaptosomes (low micromolar range) was about 23-fold less potent than that of deltamethrin. Compared to dopamine, the EC_{50} for heptachlor-induced release of GABA and glutamate from striatal synaptosomes were significantly greater (ca. 7- and 13-fold, respectively). Serotonergic terminals were the least sensitive of the nerve terminal types tested for heptachlor-evoked release and were, approximately 23-fold less sensitive than striatal dopaminergic terminals (Table 1).

An effect on dopamine release was also observed, in vitro, with chlorpyrifos (Fig. 5). Synaptosomes preloaded with dopamine were induced to release label at micromolar concentrations of chlorpyrifos, and the EC_{50} for this compound was calculated to be 3.8 ± 1.3 mM. The release was also complete, and showed an excellent fit to a sigmoidal model ($r^2 = 0.998$). No experiments with chlorpyrifos were attempted using other neurotransmitters.

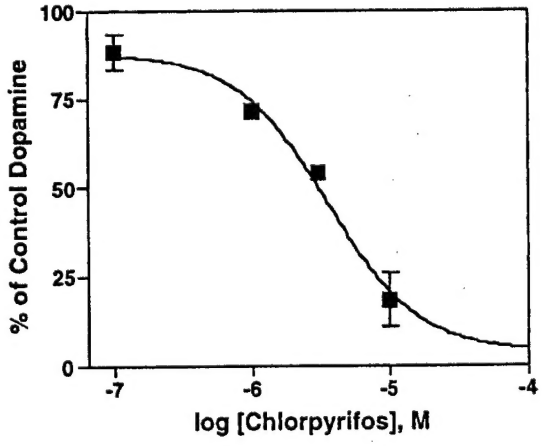

Fig. 5. Ability of chlorpyrifos to cause dopamine release from striatal synaptosomes. Symbols represent means of three determinations with bars equal to the S.E.M. Absence of bars means that the S.E.M. was less than the size of the symbol.

DISCUSSION

The ability of insecticide exposure to increase dopamine transport reflects a potent action, in vivo. We demonstrated this effect previously for the pyrethroid insecticide deltamethrin, which increased dopamine uptake by 70% following three doses of 6 mg/kg (Kirby et al., 1999). Similarly, permethrin at 1.5 mg/kg increased significantly the maximal transport of dopamine, and this dose is about three orders of magnitude below the mouse i.p. LD_{50} for this compound (Gray and Soderlund, 1985). Moreover, technical permethrin is a mixture of four (1*R*, *S-cis* and 1*R*, *S-trans*) isomers, only one of which (1*R-cis*) causes lethality in mammals (Casida et al., 1983). If the 1*R-cis* isomer alone is responsible for the upregulation, it comprises only 25% of the applied dose, and it was therefore present at about 0.4 mg/kg. A potent enhancement of uptake also occurs following treatment with heptachlor, where 6 mg/kg represents about 4% of the LD_{50} dose (145 mg/kg by i.p. injection; Cole and Casida, 1986). We assume that the observed increase in dopamine uptake was compensatory for increased levels of free synaptic dopamine, in vivo, and that balanced neurotransmission was maintained by increased expression of the transporter.

A greater abundance of DAT protein was confirmed in Western blots at doses of 0.8 and 1.5 mg/kg of permethrin. The apparent greater expression at 0.8 mg/kg, where transport was not significantly upregulated, can be ascribed to different responses of different cohorts of mice, and the neurochemical effects observed vary from group to group with respect to dose. Previous work has shown that heptachlor-dependent increase in dopamine transport was also accompanied by an increase in DAT protein labeling in Western blots of striatal membranes (Miller et al., 1999). Interestingly, we observed in the present study two labeled bands, instead of only one, which is more typical (Miller et al., 1999). These two closely spaced bands may represent different glycosylated forms of the DAT, since it is known to possess four consensus glycosylation sites (Hitri et al., 1994). Alternatively, they may be an artifact of tissue or blot processing.

Because persistent effects on DAT expression occur at low doses, it appears to be a sensitive index of subclinical toxicant insult and should be investigated further as a biomarker of environmental toxicant exposure. In addition, a recent study by Lee et al. (2001) observed an interaction between the DAT and α-synuclein in cultured neurons. This interaction led to increased dopamine uptake and dopamine-induced cellular apoptosis, and would provide a mechanism whereby increased DAT expression by insecticides could play role in the development of PD.

For both permethrin and heptachlor, transport declined at doses greater than those causing a maximal induction of dopamine uptake. This decline most likely occurred from the inability of the synaptosomes to retain dopamine, rather than a down regulation of the DAT. For both compounds, there is evidence of cell stress occurring at higher doses. In mice treated with doses ≥12.5 mg/kg permethrin, less activity was present in a synaptosomal MTT dehydrogenase assay (Karen et al., 2001), which is a measure of mitochondrial function (Carmichael et al., 1987). Similarly, polarigraphic measurements of mitochondrial respiration were reduced in striatal synaptosomes from mice treated with ≥25 mg/kg heptachlor (Kirby et al., 2001). In contrast to permethrin and heptachlor, striatal dopamine uptake was not upregulated at any dose of chlorpyrifos tested. In fact, at the highest dose (100 mg/kg), dopamine transport V_{max} was slightly decreased. As was the case for elevated doses of permethrin, treatment with 100 mg/kg chlorpyrifos significantly depressed MTT dehydrogenase activity in ex vivo synaptosomes, suggesting chemically-induced nerve terminal stress (Kirby et al., 2001).

Whereas heptachlor increased dopamine uptake in the striatum, no change in maximal rates of serotonin uptake was detected in the cortex. There was an upregulation of serotonin transport following permethrin treatment, but only at doses at least 30-fold greater than that required to upregulate the DAT. The lack of comparable effect on serotonin uptake by insecticides suggests less disruption of cortical serotonergic pathways in vivo, since compounds that either block uptake (fluoxetine; Hrdina and Vu, 1993) or inhibit synthesis of serotonin (*p*-chlorphenylalanine; Rattray et al., 1996) will affect the expression of serotonin transporter in rat cortex.

Specificity in neurotransmitter release assays is evident in the greater sensitivity of nigrostriatal dopaminergic nerve terminals to insecticide-evoked release than those of either glutamatergic or GABAergic projections to the striatum, or serotonergic terminals in the cortex. Pyrethroids and organochlorines may affect release through an action on sodium channels (Kirby et al., 1999) and calcium ion flux/homeostasis (Yamaguchi et al., 1979, 1980), respectively. The greater potency of insecticide-induced dopamine release, in vitro, is mirrored in the preferential ability of permethrin or heptachlor treatment to upregulate dopamine transport in ex vivo synaptosomes from C57 mice.

The mechanism of dopamine release by chlorpyrifos is not obviously related to its well-described action as an anticholinesterase, and we did not test its efficacy against other transmitters. However, Dam et al. (1999) found that this compound released about 20% of labeled norepinephrine from whole rat brain synaptosomes at 50 μg/ml (142 μM). Species, tissue, or transmitter selectivity could contribute to this difference. The release of norepinephrine was not antagonized by blockers of muscarinic (atropine) or nicotinic receptors (mecamylamine), so the mechanism remains unknown. The lack of DAT upregulation by chlorpyrifos treatment suggests that the release effect of this compound was not sufficient to induce transporter expression at the doses tested, or perhaps was interfered with by other poisoning processes.

Selective effects on dopaminergic nigrostriatal pathways is also observed with the mitochondrial poison, rotenone (Ferrante et al., 1997; Betarbet et al., 2000), and have been ascribed to a constitutive metabolic deficiency of nigral neurons (Marey-Semper et al., 1993). This characteristic might play some role in the greater effect of different insecticide classes having different modes of action on striatal dopamine release, although other differences in the structure or function of the release machinery for different transmitter types might also be involved.

A role for excessive release of dopamine as a neurotoxic mechanism in PD is supported by other experimental evidence. The toxic nature of elevated dopamine levels has been shown following in vivo injection (Filloux and Townsend, 1993) and through exposure to cultured neurons, in vitro (Ziv et al., 1994). Similarly, the compounds reserpine and tetrabenazine cause release of neurotransmitter from nerve terminals (Mahata et al., 1996), deplete dopamine and other monoamine levels in brain (Bowman and Rand, 1980), and Parkinsonism is a common side effect of their use as drugs in humans (Montastruc et al., 1994). Insecticides may manifest similar effects.

Although these studies have shown specific effects of insecticides on the nigrostriatal pathway, we have failed to demonstrate a reduction in dopamine titers

following heptachlor, chlorpyrifos, or permethrin treatment (Kirby et al., 2001; Karen et al., 2001). Loss of dopamine is a cardinal sign of PD (Hornykiewicz and Kish, 1987) and can reflect changes in both cellular levels of dopamine and cell loss in the nigrostriatal pathway. In previous studies, we measured the total amount of striatal dopamine by HPLC (Bloomquist et al., 1999; Karen et al., 2001). This technique may have missed incipient effects of insecticides on dopamine levels, because natural loss of dopaminergic neurons in aging is countered by an increase in dopamine synthesis by the remaining neurons (Tatton et al., 1991). In our studies, the lack of significant effect on dopamine titers by insecticides does not support a rapidly developing Parkinsonism following short term exposures to these compounds. Longer term studies, especially those at environmentally relevant exposures and in combination with other susceptibility factors (e.g. mitochondrial compromise), should be undertaken to reveal any effects of insecticides on striatal dopamine levels under these conditions.

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